Vol. 52, 1974

Acta Haematologica

International Journal of Haematology
Journal International d'Hématologie
Internationale Zeitschrift für Hämatologie

Official Organ of the European Division of the International Society of Haematology

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Acta Haemat. 52: 1–7 (1974)

Value of the Combined Cytological and Cytochemical Classification in the Management of Acute Childhood Leukemia

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Abstract. 50 leukemic patients were analyzed in a retrospective study in regard to their cytology and cytochemistry at the time of diagnosis. They were classified as ALL PAS type, SCL and [illegible] type and AML peroxydase type. The results show that the combined cytological and cytochemical classification of childhood leukemia give more information about the expected course of a newly diagnosed leukemia in regard to duration of first remission and survival time and about its proper treatment.

Key Words
Childhood leukemia
Cytochemistry
Leukemia classification

The acute leukemia of childhood is classified according to cytological and/or cytochemical criteria [1–3]. At the present time it seems that cytology alone gives enough information for the diagnosis, classification and proper treatment of the different leukemias. The more time-consuming cytochemistry technique is considered to be of no more value to these aspects than a simple stained bone marrow smear. A retrospective study of our leukemias over the past 5 years was done in order to answer the following question: Does one get more information about the expected course of a leukemic patient in using cytological and cytochemical techniques at the time of diagnosis?

Material and Methods

[illegible] leukemic patients over the period [illegible]–[illegible] met the criteria used for this study. The age at the time of diagnosis ranged between [illegible] months and 11 years.

The bone marrow smears were stained by the method of MAY GRÜNWALD GIEMSA. The cytological diagnosis was made by one of us without knowledge of the cytochemical diagnosis or the patients clinical course. The cytochemical classification was done in a similar way, the one doing it did not know the cytological diagnosis or the patients clinical course. The cytological criteria used based on FRAUMENI *et al.* [4] were:

(1) Acute lymphoblastic leukemia (ALL): the nucleus of the blasts is round, the chromatin shows some clumping, narrow indentations may be seen, contains one or no nucleoli. The cytoplasm is very scanty. The trend of differentiation goes toward lymphocytes.

(2) Stem cell leukemia (SCL): the nucleus of the blasts is round, the chromatin net fine, contains 1–2 nucleoli, no indentations. The cytoplasm is scanty to moderate. There is no trend of differentiation to be seen.

(3) Acute myelogenous leukemia (AML): the nucleus of the blasts is round, chromatin net fine, contains 2–3 nucleoli, may show wide indentations. The cytoplasm is moderate, may contain Auer rods. The trend of differentiation goes toward the myelocytic cell (granules in the cytoplasm).

Cytochemical methods and classification. PAS technique according to HOTCHKISS [5], Sudan black technique according to SHEEHAN and STOREY [6], peroxydase technique according to SCHAEFER and FISCHER [7], naphtol AS-D-chloroacetate-esterase technique according to MOLONEY *et al.* [8], naphtol AS-acetate esterase and α-naphtylacetate esterase technique according to LÖFFLER [9], naphtol AS-acetate esterase technique using NaF according to SCHMALZL and BRAUNSTEINER [10]. PAS type: positive with PAS, slightly positive with the nonspecific esterase technique. Undifferentiated type: slightly positive with the nonspecific esterase technique, negative with the other techniques. Peroxydase type: positive with the Sudan black B, peroxydase and occasionally with the NAS-D-chloroacetate esterase technique. Nonspecific esterase type: strongly positive with the nonspecific esterase technique.

Chemotherapy. 1967–1968 remission induced with vincristine and prednisone or purinethol and prednisone. Maintenance therapy consisted of purinethol or methotrexate. 1969–1970 daunomycin was added to the above mentioned drug combination for remission induction. Reinduction courses with vincristine, daunomycin and prednisone were introduced in the otherwise unchanged maintenance therapy. 1971–1973 the regimen was adjusted by and large to the one recommended by AUR *et al.* [11]. We consider a complete remission if there are no clinical symptoms in regard to the leukemia, no blasts in the peripheral blood and less than 2% blasts in the bone marrow.

Results

Of the 50 leukemias studied, 24 were classified cytologically as ALL, 17 as SCL and 9 as AML. Cytochemically, 27 were classified as PAS positive, 15 as undifferentiated type and 8 as peroxydase type (table I). Table II shows the correlation between the cytological and cytochemical classification. The best correlation with 79.1 and 70.4% is obtained be-

Table I Types of acute leukemia using cytological and cytochemical criteria

Type	n	%
ALL	24	45
PAS	27	54
SCL	17	34
Undifferentiated	15	30
AML[1]	9	18
Peroxydase[1]	8	16
Total	50	100

[1] One case of promyelocytic leukemia

Table II Correlation between cytological and cytochemical classification

Type	n	Correlation		No correlation	
		n	%	n	%
ALL	24	19	79.1	5	20.9
PAS	27	19	70.4	8	29.6
SCL	17	9	52.9	8	47.1
Undifferentiated	15	9	60.0	6	40.0
AML	9	5	55.6	4	44.4
Peroxydase	8	5	62.5	3	37.5
		33	66.0	17	34.0

tween ALL and PAS type respectively. It lies slightly above 50% between SCL and the undifferentiated type as well as in the AML and the peroxydase type. The overall correlation is 66%.

Table III presents the clinical and hematological findings of our leukemia patients at the time of diagnosis. The correlating cases of ALL and the PAS type do not have any organomegaly in 92%, there is no marked leukocytosis in 79%. In the correlating cases of SCL and the undifferentiated type the percentage without organomegaly is 33.3%, 55.6% do not have a marked leukocytosis. The difference in these parameters between the two classification groups is best seen in the correlating cases. The cor

Table III. Clinical and hematological findings (%) at the time of diagnosis

Type	n	Without organomegaly	With organomegaly	Leucocytes decreased normal or slightly increased	Leukocytoses (>12 000 /µl)
ALL	24	83.3	16.7	70.8	29.2
Correlating cases[1]	19	*94.7*	*5.3*	*79.0*	*21.0*
PAS	27	92.6	7.4	77.8	22.2
SCL	17	58.8	41.2	64.7	33.5
Correlating cases[1]	9	*33.4*	*66.6*	*55.6*	*44.4*
Undifferentiated	15	46.6	53.4	53.3	46.7
AML	9	77.7	22.3	55.6	44.4
Correlating cases[1]	5	*60.0*	*40.0*	*60.0*	*40.0*
Peroxydase	8	62.5	37.5	50.0	50.0
ALL-undifferentiated	4	2	2	2	2
SCL-PAS	6	5	1	5	1
SCL-peroxydase	2	2	0	1	1
AML-undifferentiated	2	2	0	1	1
ALL-peroxydase	1	1	1	0	1
AML-PAS	2	2	0	1	1

Organomegaly: liver and/or spleen reaching down to the height of the umbilicus, excessive enlargement of the lymph nodes, tumor-like enlargement of organs not belonging to lymphoid system such as testes, parotis gland, etc.

[1] In regard to cytology and cytochemistry.

relating cases AML-peroxydase type group show no organomegaly in 60%, no marked leukocytosis is found in 60%. Added to the table is an analysis of the cases which do not correlate in their cytology and cytochemistry. The highest discrepancy is found between ALL and the undifferentiated type and between the SCL and the PAS type.

Table IV presents the mean duration of first remission and mean survival time in regard to cytology and cytochemistry. The longest duration of first remission is met in the ALL-PAS type group with 24.2 months, the shortest in the AML-peroxydase group with 3.6 months. The SCL-undifferentiated type group lies between these two groups. In regard to the mean survival time the ALL-PAS-type group is best again with 29.7

Table III. Mean duration of first remission and mean survival time

Type	n	Mean duration of first remission, months	Mean survival time, months	Number surviving more than 3 years
ALL	28	22.6	29.3	7
Correlating cases[1]	19	24.4	29.9	7
PAS	24	22.0	29.8	9
SCL	18	10.0	19.7 (n=13)	2
Correlating cases[1]	8	8.2	13.7 (n=7)	0
Undifferentiated	14	12.3	13.2 (n=12)	1
AML	7	9.1	16.3 (n=6)	2
Correlating cases[1]	4	5.4	17.2	1
Peroxydase	7	6.3	14.1	1
Undifferentiated ALL	3	14.6	23.0	
Peroxydase SCL PAS AML	5	13.4	30.0	2

The number of cases do not correlate with the previous one, we excluded patients dying of infection but in remission and also lost a few patients who did not return to our clinic again.

[1] In regard to cytology and cytochemistry.

months, second is the AML-peroxydase-type group with 17.2 and worst the SCL-undifferentiated type group with 13.2 months. All these differences are again best seen in the correlating cases. An analysis of the noncorrelating cases is added to the table.

Discussion

The distribution pattern of the leukemias using cytological and cytochemical criteria is similar to the one found by Fastaerst et al. [4]. ALL predominates with almost 50%, followed by SCL with about 30% and AML with about 15%. Since the cytological and cytochemical data are

very similar within each group, one could agree that the more time consuming and more expensive cytochemical examination of bone marrow smears is not a necessary procedure in the management of childhood leukemia

The further analysis shows that the rate of correlation between the two methods is 70–80% in the ALL-PAS type group and 50–60% in the two remaining groups The highest number of discrepancy between cytology and cytochemistry is found within the ALL-PAS group and SCL-undifferentiated type group Most investigators consider these two groups as one the ALL group The data in regard to mean duration of first remission and mean survival time do seem to justify this separation There is a marked difference in these parameters between these two groups, this is best demonstrated in the cytological and cytochemical correlating cases Our data also show that the so-called 'high risk' leukemia cases [12] are predominantly found in our second group, these are cases with organomegaly and marked leukocytosis We therefore draw the conclusion that leukemias which belong to the second group should be considered as high-risk cases A high PAS positivity at the time of diagnosis – more than 30% – lets one expect a longer lasting first remission This is in accordance with the findings of other authors [13] One should also mention that those which lasted longest correlate in cytology and cytochemistry

Taking the data obtained for mean duration of first remission and mean survival time for the non-correlating cases we realize that they do not fit to any of the correlating ones Our interpretation for this finding is speculative We consider the correlating cases of the ALL-PAS type and SCL-undifferentiated type group as the two end points of one developing line The non-correlating cases lie somewhere in between these two points The development in regard to their morphological and cytochemical properties is not parallel, which finds its expression in the difficulties of classifying them cytologically and cytochemically The same speculation could be made for SCL and AML Their clinical course also lies somewhere between that of ALL and SCL. As a practical consequence *we also consider the non-correlating cases as high risk leukemia*

Summarizing we consider that the separation of the ALL group into two groups is of prognostic and perhaps therapeutical value, since the second group may be thought of as a high risk group Furthermore our data show that one should use cytology and cytochemistry together in classifying the leukemias it helps to classify them in a safer way and to pick out additional high risk cases the non-correlating ones

Acknowledgements

We should like to express our thanks to the Lilly Co which supported this study. We also thank Dr. SCHMALZL from the Internal Medicine Department for his help and advice in regard to the cytochemical studies.

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Acta Haemat 52 8–13 (1974)

Periodic Acid-Schiff Reaction, a Useful Index of Duration of Complete Remission in Acute Childhood Lymphocytic Leukemia

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Key Words
Acute lymphocytic leukemia
Childhood leukemia
Cytochemistry
Leukemia relapse rate
PAS reaction

Abstract The bone marrow of 22 untreated children with acue lymphocytic leukemia were examined cytochemically with periodic acid Schiff (PAS) reaction PAS positivity was correlated with relapse rate and duration of complete remission 18 months after achieving remission 10 of 12 patients with strong PAS-positive marrow at the time of diagnosis are still in complete remission in contrast to only 3 of 10 patients with weak PAS-positive marrow Of the 10 patients with weak PAS positivity one did not achieve remission five developed complete (central nervous system, CNS, and hematologic) relapse and one relapsed in the CNS Of the 12 patients with strong PAS-positive initial marrow, 10 remain in complete continuous remission one had hematologic relapse and one developed meningeal leukemia From these data we conclude that PAS positivity is directly related to the duration of initial remission in children with acute lymphocytic leukemia

The specific diagnosis of acute leukemia depends upon the identification of the predominant immature cell in the peripheral blood and in the bone marrow From epidemiologic studies the differentiation of cell types in acute childhood leukemia has been emphasized recently Fraumeni *et al* [3] described eight cell types of acute leukemia Mathé *et al* [6] subdivided acute myeloblastic, lymphoblastic and monoblastic leukemia in various subtypes and described an association between prognosis and cytological varieties

The identification of the cell types of leukemia on cytomorphologic features may be difficult Therefore, cytochemical methods were developed for the classification of different cell types in leukemia Further-

more, cytochemistry – the PAS reaction – has also been used to establish a correlation between prognosis and cytochemical findings. Laurie [5] reported in 1968 and Vowels and Willoughby [8] in 1973 that strong PAS positivity appeared to be related to longer first remission and survival in acute childhood lymphocytic leukemia. This retrospective study of 22 consecutive untreated children entering the same study from September 1 to December 31, 1971, at St. Jude Children's Research Hospital, was undertaken to investigate this point further.

Material and Methods

Bone marrow smears of 22 untreated children with acute lymphocytic leukemia (ALL) were examined cytochemically with Sudan black B staining, peroxidase and periodic acid Schiff (PAS) reaction. Marrow smears taken before the beginning of treatment were stained with PAS reaction and the percentage of PAS-positive lymphoblasts was obtained. The PAS positivity in leukemic blast cells was additionally assessed semiquantitatively according to Hayhoe [4]. The sum of 100 individual cell ratings gave the total PAS score in the blast cells from each specimen. The possible range varied from 0 to 400. According to Laurie [5] patients with less than 40% PAS-negative blast cells were considered as strong PAS positive and patients with greater than 55% negative blast cells as weak PAS positive. Sudan black B staining was used by the technique of Sheehan and Storey [7], the peroxidase reaction as recommended by Hayhoe [4].

Mode of therapy has been reported in detail [1]. For remission induction the patients received vincristine weekly and prednisone daily for 4–6 weeks. After achieving remission the patients were given prophylactic central nervous system (CNS) therapy. Maintenance therapy consisted of 6-mercaptopurine daily, methotrexate and cyclophosphamide weekly, all given by mouth.

Results

The initial findings of the 22 patients at the time of diagnosis are given in table I. The proportion of PAS-negative blast cells ranged from 10 to 97%, the total PAS score from 8 to 320. Sudan black and peroxidase reactions were negative in all 22 patients. Ten patients had a high proportion of PAS-negative lymphoblasts whereas 12 had a low proportion in their initial marrow. Of the 10 patients in the weak PAS-positive group, one did not achieve remission and six have relapsed. Complete relapse (hematologic and CNS) occurred in 5 patients, only CNS relapse in one. Of the 12 patients in the strong PAS-positive group nine remain

Table I Features at diagnosis of 22 consecutive untreated children with ALL

Case No	Sex	Age years/months		Spleen/liver enlargement cm	Mediastinal mass	Leukocyte count × 1000/μl	PAS total score (pos. blasts %)
1	M	10	10	5 5	+	105	35 (25)
2	M	15	2	0/0	+	1 4	147 (30)
3	M	1	11	2/5	−	5 1	[illegible] (29)
4	M	4	8	0/0	−	4 3	277 (78)
5	F	12	11	0/0	−	10 2	53 (25)
6	M	3	9	0/0	−	16.2	192 (62)
7	M	9	7	7/4	−	15 3	162 (55)
8	F	3	5	0/0	−	7 1	178 (69)
9	M	10	3	0/0	−	4 9	256 (79)
10	F	5	5	0 7.5	−	3 3	69 (55)
11	F	2	10	0/3 5	−	129	95 (40)
12	F	9	5	0/0	−	5 1	68 (35 5)
13	M	11	10	4 5/3	+	182	58 (30)
14	F	10	1	7/8	−	4 3	241 (77)
15	M	1	1	7 5 6 5	−	42 2	9 (3)
16	F	3	5	4/7	−	7 5	139 (55)
17	M	3	1	0/0	−	4 0	19 (16)
18	M	4	5	4 4	−	6 9	320 (90)
19	F	13	3	0/0	−	3 6	8 (4)
20	M	2	11	2/2	−	7 0	99 (60)
21	F	14	2	0/0	−	2 1	212 (85)
22	F	3	9	5/7	−	42.7	272 (79)

in complete continuous remission for 16–23 months, one relapsed in the bone marrow, one in the CNS and one expired in initial complete remission of viral myocarditis and disseminated varicella

The relation of the PAS positivity in the marrow before any antileukemic therapy was initiated and the subsequent course of all the 22 patients is shown in figure 1 The median duration of complete remission in the strong PAS-positive group is 18 5 months, the median duration of complete remission in the weak PAS-positive group is 10 9 months ($p<0.02$) 18 months after achieving remission 30% of the patients in the weak PAS-positive group remain in remission compared to 75% in the strong PAS-positive group (fig 2)

High initial blast count, hepato- and/or splenomegaly more than 5 cm was observed as often in the weak as in the strong PAS-positive group

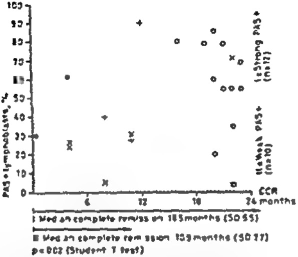

Fig. 1 Relation between percentage of PAS + lymphoblasts of 22 untreated patients with ALL at diagnosis and the subsequent course of their disease. C = Induction failure, ● = expired in complete remission, x = hematological relapse, + = CNS relapse, ○ = complete remission

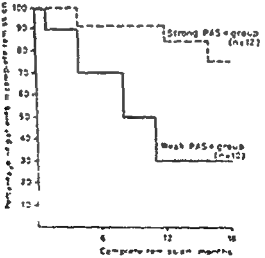

Fig. 2 Percentage of patients in strong and weak PAS + groups in complete remission.

Table II

Initial lymphoblasts in the peripheral blood mediastinal mass hepato- or splenomegaly in the *weak PAS + group* (n = 10 patients)	
4 patients c	>5 000 lymphoblasts/µl (40%)
3 patients c	mediastinal mass (30%)
3 patients c	hepato- and/or splenomegaly 5 cm or 5 cm (30%)
Initial lymphoblasts in the peripheral blood mediastinal mass hepato- or splenomegaly in the *strong PAS + group* (n = 12 patients)	
4 patients c	>5 000 lymphoblasts/µl (35%)
0 patients c	mediastinal mass
5 patients c	hepatosplenomegaly (40%)

Mediastinal mass, however, was present only in the weak PAS positive group (table II)

Discussion

LAURIE [5] described in 1968 that weak PAS positivity in leukemic lymphoblasts is significantly related to high initial peripheral blood count, short duration of symptoms at the diagnosis and short first remission and survival He concluded that PAS positivity is inversely related to the rate of progression of acute childhood lymphocytic leukemia Recently, VOWELS and WILLOUGHBY [8] confirmed these findings They reported a relation between the percentage of PAS-positive blast cells at diagnosis and duration of control of the disease for the long term results of 31 patients treated with cyclic chemotherapy

BENNETT and HENDERSON [2] reported contradictory results In a 1969 review of 44 patients studied at the National Cancer Institute they found no correlation of PAS reactivity with duration of symptoms initial leukocyte count, or duration of first remission Patients studied by LAURIE and VOWELS were treated with cyclic chemotherapy whereas the patients at the NCI received combination chemotherapy

In this small series of patients treated with total therapy, a mode of combination chemotherapy relapse occurred more often in patients with weak PAS positivity Strong PAS positivity appeared to be related to longer first remissions (fig 1) High initial blast cell count, hepato- and or splenomegaly greater than 5 cm below the costal margin were not

associated more often with PAS negativity. Mediastinal mass, however, was observed only in the group of patients with weak PAS positivity (table II). These findings imply that PAS positivity is directly related to the duration of initial remission in children with acute lymphocytic leukemia and might suggest that mode of therapy found to be most suitable for patients with strong PAS positivity is not necessarily the best for patients with weak PAS positive initial bone marrow.

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Acta Haemat. 52: 14–23 (1974)

Rate of Incorporation of Tritiated Thymidine into DNA of Normoblastic and Megaloblastic Bone Marrow Cells *in vitro*

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Abstract. A normal rate of incorporation of tritiated thymidine (^{3}H-TdR) into the DNA of bone marrow cells was found in 9 iron-deficient patients and 3 patients with chronic liver disease. By contrast, the uptake was significantly depressed in 5 out of 7 patients with vitamin B_{12} or folate deficiency, in 2 of 8 patients with megaloblastic changes induced by antipurines and in 2 of 8 patients with a macrocytosis caused by alcoholism. The low rate of uptake of ^{3}H-TdR in the latter 4 cases could not be attributed to an interference with vitamin B_{12} or folate metabolism as their marrow cells showed a normal suppression of ^{3}H-TdR uptake after pre-incubation with deoxyuridine. 3 patients with a combination of iron and vitamin B_{12} deficiency showed a normal incorporation of ^{3}H-TdR/10^{3} DNA-synthesizing cells, but the mean rate of incorporation in this group was depressed by 50% following iron therapy. These 3 patients also showed a subnormal suppression of ^{3}H-TdR incorporation after incubation with deoxyuridine and this abnormality was made worse after therapy with iron. These results indicate that the disturbance in DNA synthesis which occurs in vitamin B_{12} deficiency is ameliorated by the co-existence of iron deficiency.

Key Words
Alcoholism
Drug induced megaloblastosis
Folate deficiency
Iron deficiency
Liver diseases
Thymidine incorporation
Vitamin B_{12} deficiency

Little information is available regarding the rate of uptake of tritiated thymidine (^{3}H-TdR) by bone marrow cells in various diseases. Hershko *et al.* [5] incubated marrow cells from 4 iron-deficient patients with ^{3}H-TdR for 1 h and found that the amount of radioactivity incorporated into DNA/10^{6} nucleated cells was only 20% of that incorporated by the same number of nucleated cells from normal marrow. This result suggested the possibility of a depression in the rate of DNA synthesis in iron de-

ficiency. However, the practice of relating the amount of incorporation of ^{3}H TdR to the total number of nucleated cells in a particular marrow culture can be quite misleading. This is because at any particular time only some of the nucleated marrow cells are in the DNA-synthetic phase of the cell cycle. Furthermore, the proportion of DNA-synthesizing cells in the marrow varies from one normal person to another and may be quite abnormal in patients with disturbed haemopoiesis. The only way in which the uptake of ^{3}H TdR by bone marrow cells can be meaningfully compared in different patients is by expressing the data in terms of the amount of ^{3}H TdR incorporated by a given number of DNA-synthesizing cells.

The present paper describes a study of the rate of ^{3}H TdR incorporation/10^6 DNA-synthesizing cells in human bone marrow, with special reference to marrow cells from patients with iron deficiency, megaloblastic haemopoiesis or a combination of these abnormalities. The rate of incorporation of ^{3}H TdR into the DNA of 10^6 nucleated marrow cells was first determined using liquid scintillation counting. This result was then converted to uptake/10^6 DNA-synthesizing cells using the *in vitro* ^{3}H TdR labelling index which was determined by an autoradiographic technique. The effect of pre-incubating marrow cells with deoxyuridine on their subsequent incorporation of ^{3}H TdR into DNA was also studied.

Materials and Methods

The subjects studied consisted of 9 haematologically normal patients, 9 patients with an anaemia due to iron deficiency, 7 patients with megaloblastic haemopoiesis due to vitamin B_{12} or folate deficiency, 3 patients with a combination of vitamin B_{12} and iron deficiency, 8 patients with a drug-induced megaloblastosis and 11 patients with a macrocytosis caused by chronic liver disease or alcoholism. 2 of the 3 patients who were both iron- and vitamin B_{12}-deficient and all the patients with a macrocytosis due to alcoholism or chronic liver disease had normoblastic erythropoiesis.

Freshly aspirated bone marrow was mixed with 5 ml Hanks' solution containing preservative-free heparin. A portion of each marrow aspirate was forced through a 21-gauge needle once and a 25-gauge needle twice, and the resulting cell suspension centrifuged at 1100 g for 5 min. The buffy coat was separated and resuspended in sufficient Hanks' solution to produce a cell suspension containing 5000–10000 nucleated cells/µl. Using a sample of this cell suspension, a few more dilute cell suspensions were also prepared, with nucleated cell counts between 1,500 and 5,000/µl. All nucleated cell counts were determined using a Coulter S Counter.

To 1 ml of each marrow cell suspension were added 0.5 ml autologous serum, 0.4 ml Hanks' solution and 0.1 ml of a solution containing 5 μCi ^{3}H TdR/ml (specific activity 20 000–30 000 mCi/mM). The mixture was incubated in a shaking water bath at 37 °C for 1 h. The cells were then washed 3 times in ice-cold 0.9% NaCl and finally resuspended in about 2 ml of 0.9% NaCl. The concentration of nucleated cells in this washed, labelled cell suspension was determined, and two 0.1 ml aliquots of the cell suspension were each delivered on to individual Whatman filter discs (1.9 cm in diameter, 3 mm grade). The discs were dried overnight at 37 °C, immersed in ice-cold 10% trichloroacetic acid for 20 min and then washed in 2 changes of absolute methanol (10 min each). After a brief rinse in acetone, the discs were dried and counted in 4 ml of a standard scintillation fluid, using a Packard Tricarb scintillation counter. Each disc was counted to obtain a minimum of 5 000 counts. The amount of ^{3}H TdR incorporated into the DNA of 10^3 nucleated marrow cells was calculated from the average counts per minute per disc and the total number of nucleated cells per disc.

In each patient studied, the uptake of ^{3}H TdR was determined using 3–5 marrow cell suspensions containing different concentrations of nucleated cells. The uptake of ^{3}H TdR/10^3 nucleated cells was plotted against the total number of nucleated cells in the marrow culture and, from the inverse linear relationship between these 2 parameters, the uptake of ^{3}H TdR/10^3 cells was determined for a total of 2.5×10^6 nucleated cells. All uptake data reported in this paper, therefore, relate to 2.5×10^6 nucleated marrow cells incubated for 1 h with ^{3}H TdR, at a final concentration of 0.25 μCi/ml. The uptake of ^{3}H TdR/10^3 DNA synthesizing cells was calculated by multiplying the uptake per 10^3 nucleated cells by

$$\frac{100}{LI}$$

where LI (labelling index) is the percentage of nucleated marrow cells which are engaged in DNA synthesis as judged by their incorporation of ^{3}H TdR.

The labelling indices of nucleated marrow cells were determined as follows: 0.2–0.5 ml of the marrow aspirate were added to 2 ml heparinized Hanks' solution containing 5–10 μCi ^{3}H TdR/ml, and the mixture was incubated at 37 °C for 30 min. The labelled marrow fragments were then removed with a Pasteur pipette and smeared on glass slides. The smears were air-dried and fixed in methanol for 10 min. Autoradiographs were prepared using Ilford K_2 emulsion, exposed for 2 weeks at 4 °C and developed with Ilford Phen X. Labelling indices were determined by assessing 2,000 nucleated cells in each patient. A cell was considered to be labelled if the number of overlying grains was at least twice the average background in adjacent cell free areas.

The marrow suspension with the highest cell count was used to study the effect of pre-incubation with deoxyuridine on the subsequent incorporation of ^{3}H TdR. Marrow cultures were set up using 1 ml of the cell suspension, 0.5 ml of autologous serum, 0.2 ml Hanks' solution and 0.2 ml of Hanks' solution containing 1 μM 2-deoxyuridine/ml. The culture was incubated at 37 °C for 1 h before the addition of 0.1 ml of a solution containing 5 μCi ^{3}H TdR/ml. The uptake of ^{3}H TdR was then determined as described above.

Results

Preliminary studies indicated that the incorporation of ^{3}H-TdR into the DNA of bone marrow cells increased linearly over a period of 1 h. The 1-hour uptake of ^{3}H TdR can, therefore, be used as an index of the rate of incorporation of this substance. Table I shows the ^{3}H TdR labelling indices, the rate of incorporation of ^{3}H-TdR into the DNA of 10^6 nucleated marrow cells and the rate of incorporation of ^{3}H-TdR into the DNA of 10^6 DNA-synthesizing cells in the 47 patients studied. The labelling index was above the observed normal range in 4 of the iron-deficient patients, 1 patient with drug induced megaloblastic change, 1 patient with macrocytosis due to alcoholism, 2 patients with a combination of iron and vitamin B_{12} deficiency, 4 patients with vitamin B_{12} deficiency and the 2 folate-deficient patients. The highest labelling indices were observed in vitamin B_{12}- or folate-deficient patients. The 95% confidence limits for the uptake of ^{3}H-TdR by marrow cells from haematologically normal persons were 12.0–39.3 cpm/10^6 DNA-synthesizing cells/hour. An uptake of less than 12.0 cpm/10^6 DNA-synthesizing cells/hour was found in the 2 folate-deficient patients, 3 of the 5 patients with vitamin B_{12} deficiency, 2 patients with azathioprine induced megaloblastic change and 2 patients with alcohol induced macrocytosis. The remaining cases, including all 9 iron-deficient patients and all 3 patients with a combination of vitamin B_{12} and iron deficiency showed an uptake within the normal range.

In the vitamin B_{12}- or folate-deficient group, the mean rate of uptake of ^{3}H TdR/10^6 DNA synthesizing cells was approximately half the mean value for the haematologically normal group, and the difference between these two means was highly significant ($p<0.001$). By contrast, the mean values for the rate of uptake of ^{3}H TdR in each of the 4 other groups shown in table I were not significantly different from the mean value for haematologically normal subjects. In the 3 patients with a deficiency of both vitamin B_{12} and iron, the mean uptake of ^{3}H TdR fell from 26.9 to 13.4 cpm/10^6 DNA synthesizing cells/hour after 1–4 weeks of iron therapy.

Table II shows the effect of pre-incubating marrow cultures with deoxyuridine on their subsequent incorporation of ^{3}H TdR. It is evident that a subnormal suppression of ^{3}H TdR uptake was only seen in the group of patients with vitamin B_{12} or folate deficiency and in the 3 patients with both vitamin B_{12} and iron deficiency. In the latter group, the average

Table I The uptake of ^{3}H-TdR by bone marrow cells from patients with various haematological disorders

Diagnosis	Number of cases studied	Hb g%	MCV fl	Labelling index, %: range	Labelling index, %: mean	Uptake of ^{3}H-TdR/10^{3} nucleated cells, cpm: range	Uptake of ^{3}H-TdR/10^{3} nucleated cells, cpm: mean	Uptake of ^{3}H-TdR/10^{3} DNA-synthesizing cells, cpm: range	Uptake of ^{3}H-TdR/10^{3} DNA-synthesizing cells, cpm: geometric mean
Haematologically normal	9	11.6–13.9	90–96	10.6–25.2	15.4	2.0–4.6	3.4	16.0–30.9	21.8
Iron deficiency	9	6.4–10.5	57–71	13.8–35.7	23.7	3.0–8.9	5.5	14.8–34.2	22.8
Vitamin B_{12} or folate deficiency	7[1]	4.6–13.1	97–130	26.4–75.7	45.2	2.9–11.8	6.2	7.4–19.3	10.6
Combined vitamin B_{12} and iron deficiency	3	3.9–13.3	74–113	13.8–41.8	27.2	4.3–10.7	6.5	22.8–33.3	26.9
Drug induced megaloblastosis	8[2]	9.4–15.3	106–125	12.3–36.3	20.0	1.1–7.8	3.7	9.0–35.9	17.5
Macrocytosis due to alcoholism or chronic liver disease	11[3]	9.0–18.1	99–127	10.3–29.2	20.3	1.9–5.7	3.5	[illegible]3–37.2	17.7

[1] 2 of these patients were folate-deficient.
[2] Includes 2 patients on 6-mercaptopurine, 1 patient on thioguanine and 5 patients on azathioprine.
[3] 3 of these patients had chronic liver disease.

Table II. Suppression of ^{3}H TdR uptake after pre-incubation with deoxyuridine (0.1 µg/ml of the marrow culture) for 1 h

Diagnosis	Number of cases studied	Uptake of ^{3}H TdR after pre-incubation with dU, expressed as a percentage of the uptake without pre-incubation with dU[1]	
		range	mean
Haematologically normal	9	4.0–9.0	7.1
Iron deficiency	9	2.2–8.6	6.5
Vitamin B_{12} or folate deficiency	7	20.6–54.0	34.7
Combined vitamin B_{12} and iron deficiency	3	20.0–35.7	23.3
Drug induced megaloblastosis	8	3.5–9.2	5.7
Macrocytosis due to alcoholism or chronic liver disease	11	3.5–9.8	6.3

[1] dU = Deoxyuridine

suppression of thymidine uptake was reduced from 23.3 to 36.2% after iron therapy for 1–4 weeks.

Discussion

Before ^{3}H TdR can be incorporated into DNA, it must first be converted to thymidine monophosphate (dTMP), under the influence of the enzyme thymidine kinase, and then be phosphorylated further, under the influence of two other kinases, to thymidine triphosphate (dTTP) [3]. In any cell population, the average rate of incorporation of ^{3}H-TdR into DNA will, therefore, depend on (a) the average activity of thymidine kinase per DNA-synthesizing cell, (b) the average activity of the kinases concerned in the phosphorylation of dTMP to dTTP, (c) the average size of the intracellular dTTP pool and (d) the mean rate of incorporation of dTTP into DNA (i.e., the rate of DNA synthesis). As factors other than the rate of DNA synthesis affect the incorporation of ^{3}H-TdR into DNA, the wide range of values obtained for the rate of uptake of ^{3}H TdR/10^{6} DNA synthesizing cells in haematologically normal subjects may only be partly caused by variations in the rate of DNA synthesis in different individuals.

Table 1. The uptake of ³H-TdR by bone marrow cells from patients with various haematological disorders

Diagnosis	Number of cases studied	Hb g%	MCV fl	Labelling index, % range	Labelling index, % mean	Uptake of ³H-TdR/10⁶ nucleated cells, cpm range	Uptake of ³H-TdR/10⁶ nucleated cells, cpm mean	Uptake of ³H-TdR/10⁶ DNA-synthesizing cells, cpm range	Uptake of ³H-TdR/10⁶ DNA-synthesizing cells, cpm geometric mean
Haematologically normal	9	11.0–13.9	80–90	10.6–25.7	19.4	2.0–4.0	3.4	16.0–30.9	21.8
Iron deficiency	9	6.4–10.5	57–71	13.8–35.7	21.1	4.0–8.9	5.5	14.8–34.7	22.8
Vitamin B12 or folate deficiency	11*	4.6–13.1	97–130	20.4–75.1	41.2	2.9–11.8	6.2	7.4–19.3	11.0
Combined vitamin B12 and iron deficiency	3	3.9–13.3	74–113	13.8–41.8	21.2	4.4–10.1	6.5	22.8–37.3	26.9
Drug-induced megaloblastosis	8†	9.4–15.3	106–125	17.3–40.3	30.0	1.1–7.8	4.1	9.0–35.9	17.5
Macrocytosis due to alcoholism or chronic liver disease	11‡	9.0–16.1	97–121	10.3–29.2	20.3	1.9–5.1	3.5	11.3–37.7	17.7

* 2 of these patients were folate deficient.
† Includes 2 patients on 6-mercaptopurine, 1 patient on thioguanine and 5 patients on azathioprine.
‡ 5 of the 11 patients had chronic liver disease.

Table II Suppression of ³H TdR uptake after pre-incubation with deoxyuridine ([illegible] of the marrow culture) for 1 h

Diagnosis	Number of cases studied	Uptake of ³H TdR after pre-incubation with dU, expressed as a percentage of the uptake without pre-incubation with dU[1]	
		range	mean
Haematologically normal	9	4.0–9.0	7.1
Iron deficiency	9	2.2–8.6	6.5
Vitamin B_{12} or folate deficiency	7	20.6–54.0	34.7
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Discussion

Before ³H TdR can be incorporated into DNA, it must first be converted to thymidine monophosphate (dTMP), under the influence of the enzyme thymidine kinase, and then be phosphorylated further, under the influence of two other kinases, to thymidine triphosphate (dTTP) [3] In any cell population, the average rate of incorporation of ³H TdR into DNA will, therefore, depend on (a) the average activity of thymidine kinase per DNA synthesizing cell, (b) the average activity of the kinases concerned in the phosphorylation of dTMP to dTTP, (c) the average size of the intracellular dTTP pool and (d) the mean rate of incorporation of dTTP into DNA (i.e., the rate of DNA synthesis) As factors other than the rate of DNA synthesis affect the incorporation of ³H-TdR into DNA, the wide range of values obtained for the rate of uptake of ³H-TdR per 10³ DNA synthesizing cells in haematologically normal subjects can only be partly caused by variations in the rate of DNA synthesis in different individuals

Pre-incubation of normal marrow cultures with 0.1 μM deoxyuridine/ml of marrow culture depresses the uptake of ^{3}H-TdR to less than 10% of the uptake by control cultures which are not pre-incubated with deoxyuridine [4, 7, 10, 11]. This effect is probably mediated via an expansion of the intracellular dTTP pool as a consequence of the conversion of the added deoxyuridine to dTTP. The expanded intracellular dTTP pool would dilute any ^{3}H-dTTP formed from ^{3}H-TdR to a greater extent than a normal-sized dTTP pool so that an abnormally small proportion of the ^{3}H-dTTP molecules would be incorporated into DNA per hour. Furthermore, as high levels of dTTP are known to inhibit thymidine kinase activity [2, 6], the expanded dTTP pool may inhibit the conversion of ^{3}H-TdR to ^{3}H-dTTP. When compared with normal marrow cells, vitamin B_{12}- or folate-deficient marrow cells show a smaller depression of ^{3}H-TdR uptake when pre-incubated with deoxyuridine [4, 7, 10, 11]. Apart from the 7 patients with vitamin B_{12} or folate deficiency and the 3 patients with both vitamin B_{12} and iron deficiency, the remaining cases in the present study showed no abnormality in the conversion of deoxyuridine to dTTP.

The absence of a major disturbance in the progression of iron deficient haemopoietic cells through the DNA-synthetic phase of the cell cycle is indicated by the absence of appreciable numbers of such cells which are arrested after a period in DNA synthesis [13, 15]. In the present study, patients with iron-deficiency anaemia showed a normal uptake of ^{3}H-TdR/10^{3} DNA-synthesizing bone marrow cells. This finding would indicate the presence of a normal rate of DNA synthesis in the haemopoietic cells of these patients, provided that the level of thymidine kinase activity and the average size of the intracellular dTTP pool are unaltered by chronic iron deficiency.

Using a combination of quantitative cytochemistry and autoradiography, several authors have shown that a significant proportion of megaloblasts from patients with vitamin B_{12} or folate deficiency appear to be arrested in the DNA-synthetic (S) phase of the cell cycle [8, 12, 17]. However, there is still some doubt as to whether the average rate of DNA synthesis is depressed in those megaloblasts which are not arrested in the S phase. Thus, although it is likely that the high ^{3}H-TdR labelling indices of vitamin B_{12}- or folate-deficient neutrophil and eosinophil precursors result from a prolongation of the S phase caused by a depression in the rate of DNA synthesis, high labelling indices may also be produced by a shortening of the cell cycle (with a normal or prolonged S period) or an

increase in the proportion of cells in cell cycle [14, 16] Furthermore, in the single patient with untreated pernicious anaemia in whom the average duration of the cell cycle and the S phase have been determined by the *in vivo* labelled mitoses technique, both these parameters appeared to be essentially normal [9] The present study clearly demonstrates that the rate of incorporation of ³H-TdR into the DNA of DNA-synthesizing marrow cells is significantly depressed in some patients with vitamin B_{12} or folate deficiency The mean value for the rate of uptake of ³H-TdR/10³ DNA-synthesizing vitamin B_{12}- or folate-deficient marrow cells was only half that for normal marrow cells This reduction in the rate of thymidine incorporation reflects a reduction in the rate of DNA synthesis and cannot be attributed to either a reduction in thymidine kinase activity or an increase in the size of the intracellular dTTP pool In fact, thymidine kinase activity is known to be increased in vitamin B_{12}-deficient marrow cells [1], and as the methylation of deoxyuridylate to thymidylate is impaired in both folate and vitamin B_{12} deficiency, one would expect the size of the intracellular dTTP pool to be decreased rather than increased in both these deficiency states Because ³H-TdR bypasses the metabolic block which affects DNA synthesis in vitamin B_{12}- or folate-deficient marrow cells, it is very likely that the actual depression in the average rate of DNA synthesis in these cells is greater than that suggested by the thymidine uptake data

In the group of patients with macrocytosis due to alcoholism or chronic liver disease and in the group with drug-induced megaloblastosis, the mean values for the uptake of ³H TdR/10³ DNA-synthesizing marrow cells/hour were not significantly different from the mean value for haematologically normal individuals However, in each of these 2 groups, 2 patients showed an uptake below the 95% confidence limits for normal individuals, suggesting the possibility of a depression in the rate of DNA synthesis in some cases As all 11 patients with a macrocytosis due to alcoholism or chronic liver disease and all 8 patients with drug induced megaloblastosis showed a normal suppression of ³H-TdR uptake after pre incubation with deoxyuridine, the low rate of incorporation of ³H-TdR in the 4 cases in these 2 groups cannot be attributed to an interference with vitamin B_{12} or folate metabolism

Since thymidine uptake data are very likely to underestimate any depression in the rate of DNA synthesis in vitamin B_{12} deficiency, the significance of the normal rate of uptake of ³H-TdR/10³ DNA-synthesizing cells in the 3 patients with a combined vitamin B_{12} and iron deficiency is

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Acta Haemat 52 24-28 (1974)

Inhibition of Intestinal Absorption of Folic Acid by Phenytoin

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Abstract Folate deficiency in four patients could be ascribed to anticonvulsant therapy with phenytoin Their intestinal absorption of folic acid was depressed only when phenytoin was given simultaneously Otherwise the absorption of folic acid was normal This malabsorption of folic acid is probably due to a rise in the jejunal pH produced by phenytoin It is assumed, however, that this effect of phenytoin is rather the result than the cause of folate deficiency occurring during anticonvulsant drug therapy

Key Words
Folate deficiency
Jejunal pH
Malabsorption
Phenytoin

It is now widely recognized that megaloblastic anaemia occurs in some epileptic patients treated with derivates of barbiturates or hydantoin [7] Folate deficiency was detected in these patients by the *L. casei* method [17] as well as by measuring the folate-dependent ^{14}C-formate incorporation into lymphocytes of patients in anticonvulsant drug therapy with phenytoin [6] The action of anticonvulsant drugs on folate status is not clearly understood A number of possible explanations have been proposed including (1) inhibition of intestinal folate conjugase [5], (2) inhibition of intestinal absorption of pteroylmonoglutamate [16], (3) displacement of folate from its plasma carrier protein [15], (4) interference with folate-coenzyme function [14], and (5) increased hepatic degradation of folic acid during enzyme induction by phenytoin and barbiturates [19] Although many attempts have been made to settle the question, the experimental supports of these theories are still preliminary and rather contradictory

In this study the folic acid urinary excretion test – performed by feeding 200 μg ^{3}H-folic acid by mouth followed by immediate intramuscular injection of 15 mg non-labelled folic acid [11] – was found depressed by

phenytoin in four folate-deficient patients in long-term anticonvulsant treatment. This effect of phenytoin was a reversible phenomenon occurring only in coincidence with peroral ingestion of anticonvulsant drugs.

Case Histories

Case 1. A 45-year-old woman, treated for epilepsy for 10 years with 100 mg phenytoin three times a day was admitted with a history of 6 months tiredness, loss of appetite, and a 5 kg weight loss. Her dietary intakes were irregular and poor. On physical examination she presented a normal, well nourished state. Hb was 11.6 g/100 ml, MCV, 110 fl, WBC, 5,300/μl. The blood film showed a slight macrocytosis. Serum folate was 1.1 and 0.6 ng/ml on two occasions (normal >2.0 ng/ml). Serum cobalamines were 424 pmol/l, serum iron was 103 μg/100 ml, and serum transferrin, 230 μg/100 ml. Free acid was present in the gastric aspirate. Gastrocamera examination [1] revealed a normal gastric mucosa. Serum creatinine was 0.5 mg/100 ml. The FIGLU test after histidine load gave 4 μmol/h (normal <20 μmol/h). No blood was present in the stools. Tritiated folic acid absorption gave a urinary excretion of 32.3% of oral dose administered (normal >30.0%). On repetition of the folic acid absorption test with concomitant ingestion of 100 mg phenytoin, urinary excretion was 25.4%. The patient refused marrow puncture. Before dismissal, oral folic acid therapy was commenced and at out patient control 6 months later Hb was 13.6 g/100 ml and the macrocytosis in peripheral blood had disappeared.

Case 2. A 81 year-old woman was admitted for severe anaemia. For 15 years she had been treated for epilepsy with 100 mg phenytoin three times a day. Apart from slight headache, she had no spontaneous complaints, physical examination revealed only pallor of the skin. Hb was 7.6 g/100 ml, MCV, 114 fl, WBC, 3 800/μl, platelets 270 000/μl. The blood film showed macrocytosis and anisocytosis. The reticulocyte count was 1.5%. On marrow aspiration a dry tap was obtained, crista biopsy showed that the marrow was hypoplastic, probably myelofibrotic. Serum cobalamines were 330 pmol/l, serum folate was estimated on three occasions and showed 0.5, 1.4, and 0.5 ng/ml. FIGLU excretion after histidine load was 18 μmol/h. The pentagastrin gastric stimulation test gave a normal response for free acid and intrinsic factor. Without oral phenytoin the folic acid absorption test gave an urinary excretion of 24.0%, with 100 mg phenytoin given simultaneously, only 13.6% was excreted in the urine. A small but significant increase in reticulocytes was observed after folic acid therapy. A few blood transfusions were given, and further investigations were not attempted because of the patient's age.

Case 3. A 35-year-old epileptic woman treated with 100 mg phenobarbitone twice a day for 20 years and with 100 mg phenytoin three times daily for 2 years was admitted for severe anaemia. Four weeks before admission she lost her appetite and began to vomit. On admission she was very pale and her temperature was slightly elevated. She was in a good nutritional state and dietary review failed to uncover dietary insufficiency. Hb was 6 g/100 ml, MCV, 115 fl, WBC, 3,500/μl, platelets 250 000/μl. The blood film showed macrocytosis and anisocytosis. The reticulocyte count was 1.5%. Serum iron was 195 μg/100 ml and serum transferrin,

216 μg/100 ml Serum cobalamines were 226 pmol/l and serum folate was 0.5 ng/ml FIGLU excretion after histidine load yielded 47 μmol/h Marrow aspirate was megaloblastic Free acid was present in the gastric juice A folic acid absorption test performed simultaneously with ingestion of 100 mg phenytoin gave 20 7% urinary excretion Without phenytoin the urinary excretion was 42 0% Therapy was started with 5 mg folic acid three times daily and at out patient control 3 months later all haematologic parameters including marrow were normalized

Case 4 In a 62 year old woman anticonvulsant treatment with 150 mg phenytoin twice daily was commenced after operation for a complicated scalp fracture due to a traffic accident After the operation she was unconscious and was fed by gastric intubation She was provided with a continuous catheter Hb was 11 9 g/100 ml, MCV, 96 fl, serum cobalamines 380 pmol/l Serum folate was 1 4 ng/ml and FIGLU excretion 6 μmol/h Serum iron was 65 μg/100 ml and serum transferrin 250 μg/100 ml Serum creatinine was 0 61 mg/ml With and without concomitant oral phenytoin the folic acid absorption test gave 18 and 44 8% respectively The patient recovered rapidly, and 6 months later she was taken off the anticonvulsant drug therapy

Discussion

In the first three cases, folate deficiency was clearly documented by macrocytosis in the blood films, low folate values in serum on repeated investigations, and a haematological response to folic acid therapy In case 4 all haematological parameters were normal except serum folate, which was significantly depressed below the normal level [12]

It is most likely that the anticonvulsant drug therapy resulted in latent folate deficiency in all four cases Folate deficiency was manifested by dietary insufficiency in case 1, myelofibrosis in case 2, and elevation of temperature in case 3 All of these co factors are known to release folate deficiency in an organism with depleted folate stores In case 4, anticonvulsant drug therapy was given for only 3 weeks, and haematological manifestations of folate deficiency may develop on a later occasion

When the oral test dose was given simultaneously with phenytoin, the intestinal absorption of folic acid monoglutamate was found reduced in all four cases evaluated by the urinary ^{3}H PGA test Without addition of the offending drug, absorption of folic acid was normal in cases 1, 3, and 4 and almost normal in case 2 This indicates that depression of intestinal absorption of folic acid takes place only when phenytoin is present in the intestinal tract Otherwise, intestinal absorption of folic acid is normal This agrees with the results of DAHLKE and MERTENS ROESSLER [4], who found that peroral ingested phenytoin has a temporary depressive effect on intestinal absorption of folic acid

It has been emphasized that peroral ingestion of barbiturates and phenytoin, which are very potent alkaline agents, may cause a rise in pH in the duodenal contents towards an alkaline reaction [2] As folic acid absorption predominantly occurs at pH 6 [10], the drug mediated malabsorption of folic acid may arise from an altered pH in the small intestine Experimental supports for this concepts of pathogenesis in anticonvulsant induced folate deficiency is given by ELSBORG [9], who found that folic acid absorption from rat intestines was depressed by barbiturates perfused in non buffered solutions only, and by GERSON *et al* [13], who observed the same effect of phenytoin in man

Although folic acid malabsorption may be part of the pathogenesis in drug induced folate deficiency, it is not absolutely certain that the folate deficiency originates primarily from malabsorption of folate In persons with normal folate levels, phenytoin is unable to depress the intestinal absorption of folic acid [18] Once established, folate deficiency may cause alterations in the intestinal mucosa morphology and function [3] and this gastrointestinal dysfunction may be corrected by parenteral folic acid alone [8] Therefore, the folic acid malabsorption observed may be secondary to folate deficiency originating from another mechanism. However, once established, a vicious circle develops, and in this way malabsorption of folic acid may contribute to the pathogenesis of drug induced folate deficiency

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Acta Haemat. 52 29–39 (1974)

Von Willebrand's Disease in Italy

A Study of 13 Families from a Small Area in the Province of Vicenza

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Abstract. The results of a study of 13 families with von Willebrand's disease, living in a small zone in the province of Vicenza are reported. 21 patients presented all diagnostic signs, while 25 showed only some of them. The aspirin tolerance test gave positive results in 7 out of 10 patients with initially normal bleeding time. Platelet aggregation studies intended to establish the 'threshold concentration' of ADP and adrenaline, showed an impairment of adrenaline aggregation in 5 out of 17 patients.

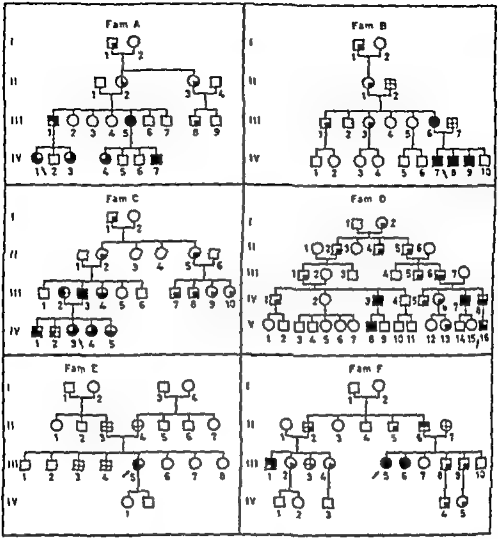
Key Words
Angiobaemophilia
Aspirin tolerance test
Bleeding disorders
Factor VIII
Platelet aggregation
Von Willebrand's disease

Von Willebrand's disease is an autosomal hereditary haemorrhagic disorder characterized by a prolonged bleeding time, a low plasma content of antihaemophilic factor (AHF) [12] and a decreased platelet adhesiveness [16]. Relatively few cases have been published to date in Italy. In 1961, OTTAVIANI *et al.* [13] reported 7 cases of angiohaemophilia A, and since then only isolated observations have been reported [9, 10, 18, 19].

This paper presents the results of investigation on 46 cases of von Willebrand's disease, coming from 13 italian families, living in a small area of the province of Vicenza. The whole population of this region numbers about 70,000.

Material and Methods

From November 1970 to December 1972, 46 patients with von Willebrand's disease were examined, 25 males and 21 females, aged between 2 and 65 years. Per-



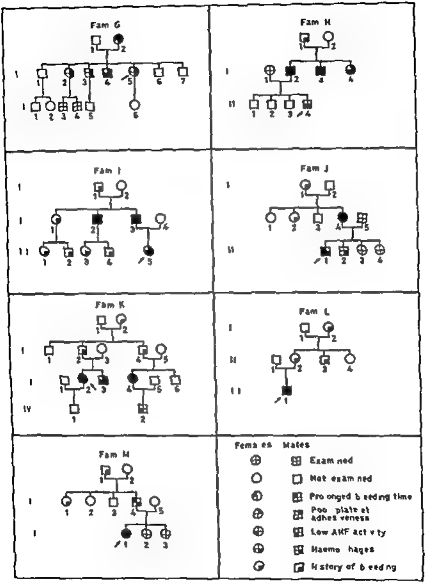
1a

Fig 1. Family pedigrees

sonal and family histories were obtained for evidence of bleeding tendencies. The research included also 22 other members of their families (fig 1a, b)

Blood was collected by disposable plastic syringes from an antecubital vein and mixed with 3.8% sodium citrate (1 part citrate to 9 parts blood). Platelet rich plasma (PRP) was *prepared by centrifuging citrated blood for 15 min at 600 rpm.* Platelet poor plasma (PPP) was prepared by centrifuging the remaining blood for 30 min at 6 000 rpm. All tests were carried out in glass test tubes, at least twice.

Bleeding time was tested according to DUKE [6] and IVY *et al* [8] modified by BORCHGREVINK [3], clot retraction in PRP according to CAEN *et al* [5], platelet adhesiveness according to SALZMAN [16], platelet aggregation according to BORN [4],


Fam G
Fam H
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Fam M
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Fig 2b

using Mustard's apparatus [11] the concentration of ADP (adenosine 5 diphosphate) and adrenaline which produced primary and secondary aggregation was noted (threshold concentration) [1] Platelet counting, Quick's time, thrombin time, factors I II, V, VIII and IX were determined as previously described [2] The aspirin tolerance test was carried out according to QUICK [15], repeating the bleeding time and platelet adhesiveness test 2 h after ingestion of 1 g aspirin ADP induced platelet aggregation was also determined daily until the secondary aggregation have reappeared (indicating the end of the aspirin effect)

Results

Table I summarizes the results of the diagnostic laboratory features of the disease, in 53 subjects Only two patients had haemorrhagic diathesis estimated as severe on account of the frequency and severity of bleeding In case IV-1 in family A, the disease was severe and the patient showed marked reduction in factor VIII activity, platelet adhesiveness was normal and bleeding time was only slightly longer than in normal subjects

In family C, 4 patients had factor VIII activity between 6–8%, but only one of these cases showed prolonged bleeding time Subject III-4 in family K showed marked lengthening of bleeding time and severe factor VIII deficiency, together with a moderate reduction in platelet adhesiveness, nevertheless, haemorrhages were only moderate

An attempt was made to find some statistical correlation between bleeding time and factor VIII activity, between bleeding time and platelet adhesiveness, between platelet adhesiveness and factor VIII, but the results were not significant ($p > 0.05$)

Table II lists the patients according to the variable expressivity of the disease 46 haemorrhagic cases were classified as definitively having von Willebrand's disease, 21 of these had all the triad lengthened bleeding time, decreased platelet adhesiveness and factor VIII deficiency The disease showed incomplete expressivity in 25 cases Four subjects showed alterations in either factor VIII, or bleeding time and platelet adhesiveness, or bleeding time and factor VIII, but did not present haemorrhages Three further subjects had mild haemorrhagic diathesis, but laboratory tests revealed no alterations

Table III gives the results obtained in the aspirin tolerance test In healthy subjects, following administration of aspirin, bleeding time was lengthened, reaching up to 12 min, and platelet adhesiveness was decreased, but only in 4 cases, down to around 8–10% Of the 15 von Wil-

Table I (continued)

Family	Case No	Bleeding time, min DUKE (1–3)	Bleeding time, min IVY (3–9)	Platelet adhesiveness % (15–60)	Plasma AHF activity % (65–150)	Haemorrhages
H	II 2	5	6	0	40	mild
	II 3	12	20	0	[illegible]	mild
	II-4	3	7	0	40	mild
	III-4	3	7	44	52	mild
I	II 2	4	11	8	38	mild
	II 3	16	22	0	15	mild
	III 5	4	8	0	36	mild
J	II-4	4	19	0	34	mild
	III 1	4	16	45	35	mild
	III 2	–	5	30	100	mild
K	III 2	5	20	0	34	mild
	III 3	–	7	0	12	mild
	III-4	14	45	7	5	mild
	IV 2	3	6	–	40	no
L	III-1	3	12	4	20	mild
M	II-4	2	12	40	70	mild
	III 1	40	–	10	28	mild

lebrand's disease patients examined, 10 showed normal bleeding time before the aspirin test Aspirin failed to affect the bleeding time in only 3 patients, whereas in the other 7 it was significantly prolonged In the remaining 5 cases, whose bleeding times were already longer than normal before aspirin, bleeding was further prolonged by the drug, although the extent of alteration was not greater than in the previous 7 patients

Platelet adhesiveness also gave noteworthy findings, in 3 cases with no initial alteration, and in 2 with reduced adhesiveness before aspirin, it was inhibited altogether

The ADP threshold concentrations showed no significant difference between healthy subjects and cases with von Willebrand's disease Adrenaline threshold concentrations (fig 2) on the other hand, were higher than normal in 5 out of 17 von Willebrand's patients These cases came from families B (IV-7), D (IV-7, IV-8, V-8), and F (III-5) Transfusion of

Table II Rate of complete and incomplete expressivity of the disease

		Number of cases	Sex M	Sex F
Willebrand patients with haemorrhages (46 cases)	complete expressivity	21	13	8
	incomplete expressivity			
	bleeding time	1	1	–
	Platelet adhesiveness	3	1	2
	plasma AHF deficiency	4	2	2
	bleeding time + platelet adhesiveness	3	2	1
	bleeding time + plasma AHF deficiency	5	3	2
	platelet adhesiveness + AHF deficiency	9	3	6
Carriers without haemorrhages (4 cases)	complete expressivity	–	–	–
	incomplete expressivity			
	plasma AHF deficiency	2	2	–
	bleeding time + platelet adhesiveness	1	1	–
	bleeding time + AHF deficiency	1	–	1
Haemorrhagic relatives without detectable laboratory defects (3 cases)		3	2	1

350 ml of plasma in patient IV-7, family B, was sufficient to lower the adrenaline threshold concentration from 1 to 0 3 μM

The duration of the aspirin effect, judging by the time taken for the second ADP-induced aggregation wave to reappear, was correlated in healthy and von Willebrand subjects with the ADP and adrenaline threshold concentrations

Platelet count, clot retraction, Quick's time, thrombin time, factors II, V, IX and X and fibrinogen were within the normal range in all patients

Discussion

The described cases of von Willebrand s disease are worthy of note as they constitute the largest case material to date in Italy Some of these cases were already reported by QUATTRIN [14] in 1949 and classified generically under the heading 'Thrombopathy'

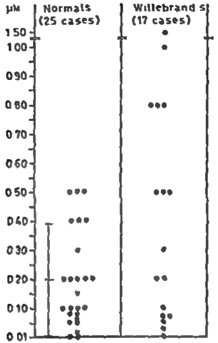

Fig 2 Adrenaline 'threshold concentrations in normals and patients with von Willebrand s disease

Prolonged bleeding time, low factor VIII activity, poor platelet adhesiveness and history of haemorrhage, were noted in 21 patients from 11 families, 19 patients in the same families and all members of family G showed some but not all these signs Only in 2 cases the disease was classified as severe on account of the frequency and severity of bleeding (patient IV-1 in family A and patient III-5 in family E) Four subjects from family C (III-2 and IV-2), family F (II-6), and family K (IV-2), who had never presented bleeding although they showed alterations of factor VIII, bleeding time and platelet adhesiveness in different combinations, were considered as not suffering from von Willebrand's disease A history of slight bleeding was reported in 3 cases (II-2 and III-3 in family F, III-2 in family J) who, however, showed no haemostatic alterations even on repeated laboratory checks No correlations were found between bleeding time and factor VIII activity, between bleeding time and platelet adhesiveness, between platelet adhesiveness and factor VIII

In 7 out of 10 patients presenting normal bleeding times, aspirin induced significant lengthening of Ivy's times reaching from 29 to 50 min

Table III The aspirin tolerance test in 10 healthy subjects and in 15 von Willebrand patients

Cases	Before			After		
	Ivy's time min	platelet adhesiveness %	plasma AHF %	Ivy's time min	platelet adhesiveness %	plasma AHF %
Healthy subjects						
1	3	50	80	5	25	n p
2	3	45	120	6	15	n p
3	4	40	110	5	30	n p
4	4	55	100	10	15	n p
5	4	35	96	6	15	n p
6	4	25	104	9	10	n p
7	5	20	88	6	15	n p
8	5	35	90	8	10	n p
9	6	40	100	12	10	n p
10	7	20	115	12	8	n p
von Willebrand patients						
G II 2	6	2	83	13	n p	n p
H II 2	6	0	40	9	n p	40
K, IV 2	6	n p	40	12	0	42
G I 2	9	15	100	31	n p	75
G II 3	5	2	62	29	n p	n p
G II-4	7	0	40	37	n p	n p
H III-4	7	44	52	30	0	50
D IV-8	7	38	10	50	0	25
D, III-6	7	19	30	36	6	35
B III-6	9	0	34	40	0	34
D V 8	11	4	40	23	0	n p
L, III 1	12	4	20	25	0	n p
A IV 1	13	21	3	25	0	18
B IV 7	18	0	30	38	0	40
H II 3	30	0	38	38	0	40

n p = Not performed

In 3 patients the test gave negative results According to TEN CATE's results [17] it might therefore be suggested that the drug acts by inhibiting the plasma factor regulating the bleeding time and platelet adhesiveness

Our own investigations into platelet aggregation were intended to establish the lowest ADP and adrenaline concentrations capable of inducing two-phases platelet aggregation In 5 out of 17 von Willebrand's patients

coming from families B (IV-7), D (IV-7, IV-8, V-8), and F (III-5) adrenaline threshold concentrations were higher than normal In patient IV-7 of family B plasma infusion corrected this abnormality showing that the defect lay in the plasma, not in the platelets themselves Platelet aggregation thus merits further investigation It may prove interesting, to study the relations between Ristocetin-induced aggregation [7] and that provoked by threshold concentrations of adrenaline

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Acta Haemat 52 40–46 (1974)

Fibrinolysis and Factor XIII

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Abstract Factor XIII levels are within the normal range in patients with pathological fibrinolysis and remain unchanged in the intense fibrinolytic state induced by streptokinase infusion Incubation of plasma with streptokinase *in vitro* results in lysis of fibrinogen as shown by a rise in the titre of fibrinogen degradation products, but levels of factor XIII remain normal Lysis of clot with factor XIII trapped within it, allows almost complete recovery of the factor XIII contained in the clot It is concluded that plasmin does not destroy factor XIII

Key Words
Factor XIII
Fibrinolysis
Plasmin

Plasmin is known to lyse a number of clotting factors in addition to fibrinogen and fibrin [3], but there is controversy as to whether plasmin affects factor XIII Some authors state that factor XIII levels are reduced in states of pathological fibrinolysis and in fibrinolysis induced by streptokinase infusion [1, 4, 5, 15] Others have reported only slightly reduced levels of factor XIII in association with fibrinolysis [7], while work in experimental animals and *in vitro* suggests that fibrinolysis does not affect factor XIII and that plasmin neither activates nor destroys factor XIII [6, 17]

We have used 3 methods to establish whether plasmin affects factor XIII We have studied the *in vitro* effects of plasmin on factor XIII in plasma and on factor XIII trapped in clot, we have estimated factor XIII in patients with presumptive evidence of fibrinolysis as shown by elevated titres of fibrin degradation products (FDP) associated with high plasminogen activator activity (short euglobulin lysis time) and we have studied the effect of streptokinase infusion on factor XIII in a patient with pulmonary embolism

Patients, Materials and Methods

Eighty hospital in patients were investigated. These fell into 4 groups: (1) 30 patients with biopsy-proven chronic liver disease, (2) 19 patients with disseminated malignancy, (3) 9 patients with leukaemia, (4) 23 patients with septicaemia, rheumatoid arthritis, malignant hypertension, lymphoma, collagen disease and severe infections. Each patient had factor XIII, euglobulin lysis time (ELT), FDP, fibrinogen and platelet count measured. Collection of blood and methods of estimation were as outlined elsewhere [10].

Patient treated with streptokinase. A 69-year-old woman was admitted with a fracture of the neck of the femur. This was treated with nail insertion under general anaesthetic. Sixteen days after admission the patient was suddenly found to be collapsed, pulseless and cyanosed and was treated with external cardiac massage and positive pressure ventilation. ECG showed changes compatible with pulmonary embolism. She was treated with heparin and warfarin but, after initial improvement, lapsed into heart failure. She was given a loading dose of 150 000 U of streptokinase in the first 30 min followed by 2.4 million U by infusion in the succeeding 24 h. Samples of blood were taken before and at timed intervals during therapy for factor XIII, ELT, fibrinogen and FDP estimation.

Arvin solution in Tris buffer was kindly prepared and supplied by Twyford Laboratories Ltd, Twyford Abbey Road, London.

In vitro *Studies*

Effect of fibrinolysis on plasma factor XIII levels. Normal fresh citrated plasma (1 ml 3.8-percent tri-sodium citrate to 9 ml of blood) was incubated with streptokinase at a final concentration of 25 U/ml. Samples were removed at timed intervals for estimation of factor XIII by measurement of ^{14}C-glycine ethyl ester incorporation into casein as previously described [10]. At the same time, samples were taken into Trasylol (100 U/ml), clotted with thrombin (5 U/ml of 0.025 M calcium chloride), the clot allowed to retract and the serum used for the estimation of FDP titre by the tanned red cell agglutination inhibition immunoassay of MERSKEY *et al.* [8]. The rise in the FDP titre with time was taken as an index of fibrinolytic activity.

Effect of fibrinolysis on factor XIII trapped within clot. Clots were prepared from fresh citrated plasma by adding (a) thrombin: 1 ml of plasma plus 0.2 ml thrombin (10 U in Tris buffer), or (b) Arvin: 1 ml of plasma plus 0.2 ml of Arvin (10 U in Tris buffer). Clots appeared within 5 sec with thrombin and 8 sec with Arvin. The tubes were left for 1 h at 37 °C. The clot was dislodged by gently tapping the side of the tube, and the tubes were then centrifuged at low speed (275 g) for 10 min. About 0.5 ml of serum was recovered from each tube and factor XIII was estimated in this. The clots were then suspended in normal saline and decanted onto discs of nylon cloth. These were placed on the funnel of a Millipore filter, washed exhaustively with normal saline and suspended in 1-ml volumes of streptokinase in normal saline (50 U/ml). Usually, the clots dissolved completely in 4–12 h. Factor XIII was estimated in the lysate.

Results

The relationship between factor XIII, FDP, and ELT in patients in whom all 3 measurements were performed on the same sample is shown in figure 1.

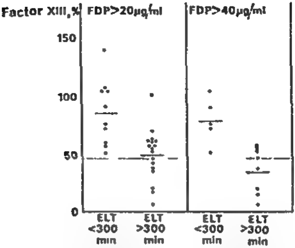

Fig 1. Relationship of factor XIII (% of normal standard plasma) to ELT in patients with elevated titres of FDP Dotted line indicates lower limit of normal for factor XIII (mean −2 SD)

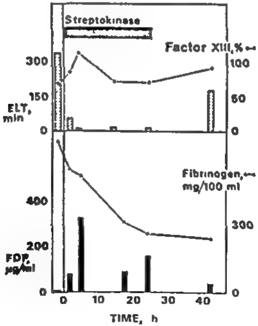

Fig. 2. Effect of streptokinase infusion on factor XIII (●), ELT (shaded columns), fibrinogen (▲), and FDP (black columns) Factor XIII activity did not fall below pre-treatment level, in the presence of an intense fibrinolytic state

Patients with elevated FDP titres (>[illegible] μg/ml) and a short or normal ELT (<300 min) have factor XIII levels within the normal range (112% of normal standard plasma pool ± 33 SD). This is also so in patients with markedly elevated FDP (>40 μg/ml) and a short or normal ELT (fig. 1). If the combination of short ELT and elevated FDP is taken as indicating enhancement of fibrinolysis, it thus appears that this is not associated with a fall in the level of factor XIII. Among the patients with elevated titres of FDP, it is only those with a prolonged ELT, i.e., those patients in whom disseminated intravascular coagulation (DIC) rather than fibrinolysis is the likely cause of the elevated FDP titre, who may have low levels of factor XIII in their plasma (fig. 1).

The effect of therapeutic fibrinolysis induced by streptokinase infusion is shown in figure 2. An intense fibrinolytic state was induced with ELT falling to below 20 min, a profound fall in the plasma fibrinogen and a marked rise in FDP. Factor XIII never fell below the pre-treatment level and even showed a slight rise initially.

The result of incubation of plasma with streptokinase *in vitro* is shown in figure 3. The titre of FDP rises with time indicating that fibrinogen is being lysed but no significant change occurs in the factor XIII level indicating that plasmin does not destroy factor XIII under the conditions used. The fall in the FDP titre observed towards the end of the period of incubation was taken to represent the formation of the late products of fibrinogen digestion to which the red cell agglutination inhibition immunoassay is much less sensitive [16].

Figure 4 shows the result of clot lysis and recovery of factor XIII from Arvin and thrombin clots. Lysis of an Arvin clot allows 80% recovery of factor XIII, which allowing for loss during washing of the clot suggests that no loss of factor XIII activity has occurred. No recovery is seen from a thrombin clot.

Discussion

The effect of plasmin on factor XIII is not established and the few statements to be found in the literature are not in agreement. Suzuki [15] states that incubation with streptok[illegible]

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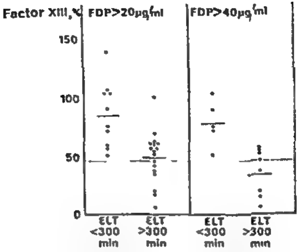

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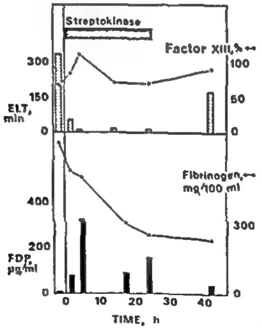

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The effect of plasmin on factor XIII is not established and the few statements to be found in the literature are not in agreement. Suzuki [15] states that incubation with streptokinase of plasma from a patient with inherited afibrinogenaemia results in a fall in factor XIII levels as measured by a clot solubility assay. A reduction in factor XIII levels in patients with 'spontaneous and streptokinase induced fibrinolysis' was mentioned by Heene [5]

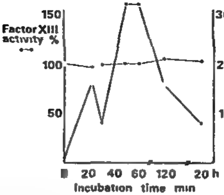

Fig 3 Effect of streptokinase on plasma factor XIII *in vitro* ● = FDP, ○ = factor XIII Fibrinogen lysis occurs as shown by the appearance of FDP, but factor XIII levels do not change

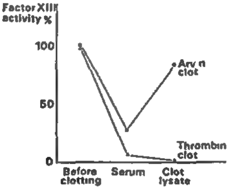

Fig 4 Recovery of factor XIII from Arvin clot and thrombin clot following lysis of clot by streptokinase

but details were not given DUCKERT and BECK [4] state that factor XIII levels fall during fibrinolytic therapy, but give no data in support BAGETT *et al* [1] found low levels of factor XIII, as measured by semiquantitative techniques, in 3 patients with 'fibrinolytic bleeding' and a rise with antifibrinolytic therapy In contrast, KOPEĆ *et al* [6] found that plasmin neither activated nor destroyed factor XIII *in vitro*, and ZUCH [17] states that injection of urokinase into rabbits results in a profound fall in fibrinogen but leaves factor XIII levels unchanged, and MANDALAKI and SCHIZAS [7] found factor XIII levels near the lower limit of normal, i e , only slightly reduced, in patients with 'primary fibrinolysis'

Our results indicate that plasmin does not destroy factor XIII Prolonged incubation of plasma with streptokinase left the factor XIII levels unchanged and *in vivo* we could find no evidence that fibrinolysis, either spontaneous as seen in patients with short ELT and elevated FDP titres, or therapeutically induced by streptokinase infusion affected plasma factor XIII levels Our results are thus in keeping with those of KOPEĆ *et al* [6] ZUCH [17] and MANDALAKI and SCHIZAS [7] The differences from the results of other authors are not easily explained DUCKERT and BECK [4] and HEENE [5] do not give sufficient details for direct comparisons to be possible BAGGETT *et al* [1] used a factor XIII assay based on clot solubility and plasma dilution which is at best semi-quantitative and in their patients, it is highly likely that factors other than fibrinolysis were responsible for the fluctuations in the factor XIII levels Perhaps the results of SUZUKI [15] that afibrinogenaemic plasma incubated with streptokinase showed a gradually decreasing ability to correct the clot solubility defect of factor XIII deficient plasma depend on the effects of breakdown products of other clotting factors

Arvin neither activates nor destroys factor XIII [2 13] and almost all the factor XIII trapped in an Arvin clot can be recovered by streptokinase induced clot lysis confirming that streptokinase does not destroy factor XIII trapped in the clot. Thrombin destroys factor XIII on prolonged incubation [13 14] and thus factor XIII cannot be recovered from a thrombin clot In DIC, both absorption on to fibrin and destruction by thrombin may contribute to low factor XIII levels but in fibrinolytic states factor XIII levels should remain normal, since thrombin is not activated and fibrin is not formed We have suggested [9] that if factor XIII is not affected by *plasmin* but is destroyed by thrombin [13 14] its levels in plasma might be normal in fibrinolytic states and low in states of *DIC* and thus, factor XIII levels might be useful in distinguishing between severe primary fibrinolysis and DIC which are thought to produce similar clinical syndromes and to occur in similar circumstances [11]

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Acta Haemat. 52 47–52 (1974)

Effect of Exercise on Platelet Count, Adhesion, and Aggregation

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Key Words
Exercise and platelets
Platelet functions
Platelet number

Abstract 15 min of strenuous exercise caused a highly significant rise in the venous platelet count without altering platelet adhesion to glass. It also produced a significant increase in the rate of the second phase of ADP- and adrenaline induced aggregation, 5-HT induced aggregation and disaggregation and collagen induced aggregation. These changes in platelet number and function following exercise should be taken into account when anti-platelet drugs are assessed in human subjects, and when changes in these parameters are sought in disease states.

Physical exercise influences several components of the haemostatic mechanism such as factor VIII [15, 16] and plasminogen activator [1]. A number of studies have shown that short periods of exercise increase the venous platelet count [2, 4, 5, 9, 17], but it has been reported that there is no change in the count after prolonged exercise [2, 14, 17]. The increased platelet count following short periods of exercise is not associated with an increase in platelet adhesiveness [2, 9], although after prolonged exercise platelet adhesion is reduced [2, 14]. Platelet aggregation after exercise has not been investigated in detail, although Ikkala *et al.* [9] noted a somewhat variable increase in the rate of aggregation induced by adenosine diphosphate (ADP) during exercise. We have studied the effect of strenuous exercise on platelet aggregation induced by ADP, adrenaline, collagen, and 5 hydroxytryptamine (5-HT). We have also re-examined the effect of exercise on the platelet count and platelet adhesion to glass using the rotating bulb method.

Materials and Methods

24 male colleagues and medical students aged 20–35, were studied. All subjects were in good health, but many were unaccustomed to strenuous exercise. The ex

[1] The authors are indebted to their subjects who exercised so strenuously and to Mrs Naomi Hoogstadt for her technical assistance.

periments were started at 2 30 p m after a light lunch at 12.30 p m After a 15 min rest a sample of citrated blood was obtained and the subjects invited to run as fast and as far as they were able to for 15 min A further sample of citrated blood was obtained and each pair of samples handled identically during all experiments

ADP trisodium salt (Koch Light Laboratories) adrenaline hydrogen tartrate and 5 HT creatinine sulphate (BDH Chemicals Ltd) and collagen (Sigma Chemical Company) were made up in Tris saline buffer (pH 7 4)

Venous blood was taken directly into 3 8% sodium citrate contained in a poly propylene syringe in the ratio of nine parts of blood to one of citrate *Platelet Counts* were performed in duplicate under phase contrast microscopy *Haematocrit* was determined in citrated blood using a microhaematocrit centrifuge

Platelet Adhesion was measured by a modification of the rotating bulb method [19] The citrated whole blood was allowed to stand at room temperature for exactly 60 min and then gently mixed An initial platelet count was obtained and two 2 ml aliquots were placed in two glass bulbs (Glass Appliances Aberdeen) and rotated for 20 min at 3.5 rpm after which a further platelet count was obtained Platelet adhesion was taken as the difference of the two counts expressed as a percentage of the pre rotation count

Platelet aggregation was measured using a Born Mark II Aggregometer (Royal College of Surgeons London) connected to a Servoscribe potentiometric recorder Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained by centrifugation started exactly 5 min after venepuncture of citrated whole blood at 375 g for 10 minutes and 1 500 g for 15 min respectively Since the rate of platelet aggregation is influenced by the time interval after venepuncture [18] aggregation was undertaken at 1 and 2 h after venepuncture and each different concentration of aggregating agent added at exactly the same time interval after venepuncture for each pre and post exercise sample of PRP

Aggregation was induced with ADP adrenaline collagen and 5 HT in aliquots of PRP which had been warmed at 37 °C for 2 min and stirred at 1 200 rpm for 1 min ADP was added to a final concentration of 4.55×10^{-6} and 4.55×10^{-7} M to obtain irreversible and reversible aggregation respectively The induction of the double wave of aggregation [11] required slightly different concentrations of ADP in different individuals but was usually $3\ 49\times10^{-6}$ M Adrenaline was used in a final concentration of $0\ 48\times10^{-4}$ M collagen in a final concentration of 0 04 mg/ml and 5 HT in a final concentration of $0\ 91\times10^{-5}$ M

The rate of aggregation was taken as the tangent to the steepest part of the aggregation curve with the optical transmission for PRP and PPP set at 0 and 100% respectively The rate of aggregation was then expressed as the change in percentage per min (Δ% min^{-1})

Results

Platelet Count was measured in 20 pairs of samples obtained from 16 subjects (4 subjects exercised twice) The platelet count increased in 18 of the 20 post-exercise samples and there was a highly significant ($p<0\ 001$) increase in the mean platelet count after exercise in both the citrated

Table I Exercise and platelet count, adhesion, and haematocrit

	Blood platelet count/μl	Adhesion, %	PRP platelet count/μl	Haematocrit, %
Before exercise	194,238 ± 9 021	37 4 ± 2.62	421 950 ± 24 749	38 0 ± 0.57
After exercise	240 713 ± 12,114	37 1 ± 2.34	520 800 ± 28,302	40 6 ± 0 49
P (paired t test)	<0 001	>0 1	<0 001	<0 001

Figures refer to means of 20 observations ± 1 SD

whole blood and PRP (table I) *Platelet adhesion* was measured in the same 20 pairs of samples of citrated whole blood, but showed no significant change after exercise (table I) *Haematocrit* was significantly increased (p<0 001) in the same 20 post-exercise samples of citrated whole blood (table I)

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Discussion

This work confirms that a rise in the venous platelet count occurs after short periods of strenuous physical exercise This rise is too large to be attributable to the small increase in venous haematocrit and occurs so rapidly that it is unlikely to be due to increased platelet production It presumably reflects an increased mobilization of platelets, although their source is uncertain It is unlikely to be solely the spleen, since post-exercise thrombocytosis may occur in splenectomized subjects [4] There is some evidence that platelets may be mobilized from the lungs by adrenaline infusion, but such platelets have been claimed to show an increased adhesiveness [10] and may, therefore, be of a different origin from those

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Table II Exercise and the rate of platelet aggregation (Δ% min^{-1})

Aggregating agent	Response	Molar concentration	1 h post venepuncture: pre-exercise	1 h post venepuncture: post-exercise	2 h post venepuncture: pre-exercise	2 h post venepuncture: post-exercise
ADP	irreversible aggregation	4.55×10^{-6}	131.6 ± 6.39	141.6 ± 5.05	116.3 ± 3.51	114.5 ± 3.39
ADP	reversible aggregation	4.55×10^{-7}	19.9 ± 4.13	18.5 ± 2.94	17.7 ± 3.11	16.7 ± 2.0
ADP	disaggregation	4.55×10^{-7}	12 ± 2.73	9.1 ± 1.91	3.8 ± 1.11	3.5 ± 1.04
ADP	1st phase aggregation	see Methods	91.2 ± 3.37	92.8 ± 2.97	74.1 ± 3.55	78.6 ± 4.15
ADP	2nd phase aggregation	see Methods	34.4 ± 3.26	49.1 ± 3.88***	15.9 ± 2.77	35.6 ± 3.67**
Adrenaline	1st phase aggregation	0.48×10^{-4}	29.2 ± 3.63	31.3 ± 3.96	43 ± 3.24	44.4 ± 4.19
Adrenaline	2nd phase aggregation	0.48×10^{-4}	57.0 ± 6.83	65.1 ± 6.53*	44.2 ± 5.7	51.8 ± 6.07*
5 HT	aggregation	0.91×10^{-5}	15.1 ± 1.73	18.5 ± 1.96*	12.9 ± 1.44	16.3 ± 2.22
5 HT	disaggregation	0.91×10^{-5}	11.2 ± 1.26	13.7 ± 1.34**	10.8 ± 2.19	11.8 ± 0.68
Collagen	aggregation	0.04 mg/ml	97.7 ± 9.72	110.1 ± 6.36	50.8 ± 8.43	77.1 ± 6.44***

Figures refer to means of 10 observations ± 1 SD. Student's test for paired data * $p<0.05$, ** $p<0.01$, *** $p<0.001$

appearing after exercise which do not BENNETT [2] has suggested that the increased number of platelets may be due to their entry into the axial blood flow from the periphery of the vascular lumen

Whatever the source of the platelets mobilized by exercise, it appears that their adhesiveness to glass is the same as those circulating under normal circumstances Both our results and those of IKKALA *et al* [9] and BENNETT [2] who used a glass bead column method, are similar Using the glass-wool filter technique, FINKEL and CUMMING [5] reported a rise in platelet adhesion after exercise at 25 °C, but not at –20 °C The significance of these results is not clear, since there was a rise in platelet count after exercise at both temperatures The rise in haematocrit after exercise would not be great enough to influence platelet adhesion in either the rotating bulb or glass bead systems [7]

The effect of exercise on some, but not all aspects of platelet aggregation is difficult to explain One possibility is that changes after exercise occur only in situations associated with the platelet release reaction, although the evidence that 5 HT induces release is inconclusive [8, 13] The lack of effect on irreversible ADP induced aggregation, which is associated with release of platelet constituents [12], may be due to the fact that so much exogenous ADP was added that any small effect of exercise on the release reaction was obscured Another possibility is that the post-exercise changes in aggregation were secondary to the increased platelet count in the PRP Such an explanation is unlikely, however, since an increase in all aspects of platelet aggregation did not occur

Our results do not confirm those of HARRISON *et al* [6] who reported that plasma instability' was increased by exercise the changes in platelet aggregation with increasing time interval after venepuncture occurred at the same rate in pre- and post-exercise PRP

Strenuous exercise can clearly influence both platelet number and function, although the mechanism of these changes is not established It is well recognized that exercise must be controlled when tests of coagulation and fibrinolysis are performed This study indicates that similar control is required when platelet number and function are measured

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Acta Haemat. 52: 53-60 (1974)

Haemoglobin Madrid β115 (G17) Alanine→Proline: an Unstable Variant Associated with Haemolytic Anaemia

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Abstract. A new unstable haemoglobin, Hb Madrid (β115 [G17] Ala → Pro), has been found in a Spanish boy suffering from a moderately severe haemolytic anaemia. This haemoglobin is the eighth example of haemoglobin instability resulting from insertion of a proline residue into a helical segment of the globin chain. The clinical and haematological features of the affected carrier are described, and the significance of substituting the normal amino acids by proline is discussed in terms of the stability of haemoglobin.

Key Words
Hb Madrid
Haemoglobinopathy
Haemolytic anaemia
Unstable haemoglobins

Many cases of haemolytic anaemia due to the presence of an unstable haemoglobin have now been described, such disorders being generally known as unstable haemoglobin haemolytic anaemias (UHHA) [5]. Instability of the haemoglobin tetramer can result from a variety of alterations in critical regions of the molecule, such as substitutions in the haem pocket or at an interchain contact, disruption of an α-helical region by introduction of a proline residue, or deletion of one or more amino acid residues can also lead to instability. All of these unstable haemoglobins lead to varying degrees of haemolysis. We report another example which, due to either disruption of an α helical region and/or weakening of an $\alpha 1\beta 1$ contact, results in a moderately severe haemolytic anaemia in the affected carrier.

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Many cases of haemolytic anaemia due to the presence of an unstable haemoglobin have now been described, such disorders being generally known as unstable haemoglobin haemolytic anaemias (UHHA) [5]. Instability of the haemoglobin tetramer can result from a variety of alterations in critical regions of the molecule, such as substitutions in the haem pocket, or at an interchain contact, disruption of an α-helical region by introduction of a proline residue, or deletion of one or more amino acid residues can also lead to instability. All of these unstable haemoglobins lead to varying degrees of haemolysis. We report another example which, due to either disruption of an α-helical region and/or weakening of an α1β1 contact, results in a moderately severe haemolytic anaemia in the affected carrier.

Methods

Electrophoretic analysis of the haemolysate was carried out on paper [8] starch gel [23] agar gel [26] and cellulose acetate [18] the latter being used to quantitate Hb A_2. Isopropanol stability tests were carried out by the method of CARRELL and KAY [4] and heat (50 °C) stability tests according to DACIE et al [10] Haemoglobin F was quantitated by alkali denaturation [1] and tests for Heinz bodies were made by incubating red cells with brilliant cresyl blue [25] Other haematological studies were carried out using standard methods [9]

Globin was prepared from the unpurified haemolysate and from the isopropanol precipitate [31] and was separated into its component chains by chromatography on carboxymethylcellulose (Whatman CM 23) [7] The pooled fractions containing the purified β and γ-chains were allowed to react with ethyleneimine in 1 M Tris/HCl pH 9 [7] and the protein was isolated by freeze-drying after removal of urea and salts by gel filtration on a 100×2.5 cm column of Sephadex G 25 (coarse) equilibrated with 0.5% (v/v) formic acid in water The α-chains were isolated in similar fashion without prior aminoethylation Preparative fingerprints of tryptic digests of the unmodified α and aminoethylated β and γ-chains were prepared [30] and stained with 0.02% (w/v) ninhydrin in acetone containing 1% (v/v) pyridine

For amino acid analysis the peptides were eluted with constant boiling HCl containing 1% (w/v) phenol for tyrosine containing peptides [28] They were then hydrolyzed in sealed tubes at 108 °C for 24 or 48 h After removal of the HCl *in vacuo*, the hydrolysates were analyzed using a Locarte amino acid analyzer Peptides which were neutral at pH 6.4 were purified by paper electrophoresis at pH 3.5 before analysis In order to determine the sequence of the first residues of the mutant peptide the latter was eluted with 0.5 M NH_4OH dried and then degraded by the dansyl Edman technique described by GRAY [12] when after two steps of degradation dansyl proline was expected as the N terminal residue the dansyl peptide was hydrolyzed for only 6 h instead of the usual 18 h

Case History

The patient is a 16-year old male of Spanish origin He was normal at birth but after 1 year he suffered from frequent attacks of jaundice pallor, and pigmenturia associated with respiratory infection At the age of 4 he was diagnosed as suffering from a congenital non spherocytic haemolytic anaemia and was treated with intermittent blood transfusions

In 1972 he was hospitalized with fever headache dyspnea jaundice pallor and pigmenturia On examination apart from obvious jaundice the only other abnormal sign was splenomegaly 15 mm below the costal margin The liver was not enlarged Hb 9.7 g/dl RBC, 3.35 10⁶/µl MCV 94 fl MCH 28.3 pg reticulocytes 33.5% WBC, 6 900/µl platelets 205 000/µl The bone marrow showed hypercellularity with a reversion of the myeloid erythroid ratio (3:7) The iron stores

were decreased, but normal numbers of sideroblasts were present. The heat stability test was positive after heating the haemoglobin at 50 °C for 1 h, precipitation was also observed after addition of isopropanol, and Heinz bodies were generated after 4 h by adding brilliant cresyl blue.

The fetal haemoglobin concentration of the patient's blood was elevated to 12.8%, and the Hb A_2 concentration, (4.3%) was also slightly raised (normal range 2.5–3.5%). Starch gel electrophoresis revealed a band in the region where free α-chains are normally found, but no other additional fraction was noted in any of the electrophoretic systems. The patient was unmarried, his father, mother and brother had no haematological abnormality.

Results of Structural Studies

The elution profile of the globin sample from the unpurified haemolysate confirmed a marked increase in the proportion of γ-chains, and hence Hb F, and also a slight increase in the proportion of δ-chains (from Hb A_2), but it was otherwise normal. The elution pattern of the globin from the isopropanol precipitate, however, indicated the presence of only α- and β-chains, both of which eluted in the normal position. The ratio, by weight, of β:α was approximately 2:1, suggesting that the β-chain was abnormal and had been preferentially precipitated by the isopropanol [2, 4].

The fingerprints of the tryptic digests of the unmodified α- and aminoethylated (AE) β-chains from the isopropanol precipitate, and the AE γ-chains from the total globin, showed no obvious abnormalities. It was therefore necessary to analyze all the tryptic peptides of the presumed abnormal β-chain from the isopropanol precipitate. It was found that all had the normal amino acid composition except βTpXIIb (β113–120), which differed from the normal in having no alanine residue and having instead one residue of proline (table I). As there is only one residue of alanine in β^{A}TpXIIb at position 115 (G17), the mutation was assumed to be β115 (G17) Ala → Pro (table II). This was confirmed by sequencing [12] the first three residues of the peptide, which gave the expected result Val-Leu-Pro, showing that proline was present at position β115. The new haemoglobin was named Hb Madrid.

The abnormal haemoglobin did not separate from Hb A on paper, starch gel, agar gel, or cellulose-acetate electrophoresis, and it seemed unlikely that it would be readily separable by other means. Therefore, to estimate the proportions of Hb A and Hb Madrid, fingerprints were

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Table I Haemoglobin Madrid, analysis of βTpXIIb (β113–120) from the isopropanol precipitate[1]

Amino acid	Molar ratio found	Ratio expected in normal βTpXIIb
Pro	0 93	–
Gly	1 13	1
Ala	0 14	1
Val	0 74[2]	1
Leu	0 96	1
Phe	0 82	1
His	2 18	2
Lys	1 13	1

[1] One amino acid residue is approximately 31 6 nmoles
[2] N terminal residue, partially oxidized by reaction with ninhydrin on paper

Table II Amino acid sequence of the tryptic peptide XIIb of the β-chain of normal haemoglobin and Hb Madrid

Residue No	113	114	115	116	117	118	119	120
Helical No	G15	G16	G17	G18	G19	GH1	GH2	GH3
Residues								
Hb A	Val	Leu	Ala	His	His	Phe	Gly	Lys
Hb Madrid	Val	Leu	Pro	His	His	Phe	Gly	Lys

prepared from tryptic digests of the aminoethylated [24] total globin and stained with dilute ninhydrin, the single spot which corresponded to the overlapping peptides β^{A}TpXIIb, β^{Madrid}TpXIIb and α^{A}TpXIIa was eluted, hydrolyzed, and analyzed, and the ratio Hb Madrid Hb A calculated from the proline alanine ratio The concentration of Hb Madrid could then be expressed as an approximate percentage of the total, taking into account the known concentrations of Hb F and Hb A_2 and was found to represent 23% of the total haemoglobin (The presence of α^{A}TpXIIa does not interfere with this calculation, since it contains no proline or alanine, however, some error could arise if the two peptides βTpXIIb exhibited differing solubilities during purification)

Table III Unstable haemoglobins resulting from proline substitutions in Hb A

Name	Position	Substitution	Clinical disorder	Reference
Genova	β28 (B10)	Leu – Pro	moderate	27
Perth (Abraham Lincoln)	β32 (B14)	Leu – Pro	severe	15 [illegible]
Duarte	β62 (E6)	Ala – Pro	moderate[1]	2
Santa Ana	β88 (F4)	Leu – Pro	severe	19
Sabine	β91 (F7)	Leu – Pro	severe	29
Southampton (Casper)	β106 (G8)	Leu – Pro	severe	[illegible] 16
Madrid	β115 (G17)	Ala – Pro	moderate	
Bibba	α136 (H19)	Leu – Pro	severe	17

[1] The propositus for Hb Duarte was also heterozygous for β-thalassaemia (see text).

Discussion

The tertiary structure of each of the haemoglobin subunits has a high α-helical content essential for maintaining a constant hydrophobic environment for the haem group. PERUTZ [20] pointed out that residues of proline, an imino acid, were unable to form an α helix and were generally found at the inter helical segments of the chain where the helix had to be broken. PERUTZ *et al.* [21] also showed that the proline residue can be accommodated in the first three residues of the helical segment, thereafter, its presence would be expected to result in disruption of the helix; this is borne out in human haemoglobin by the description of seven unstable variants (table III), all but one of which have been associated with a severe haemolytic anaemia. The finding of Hb Madrid provides another such example.

The explanation of the instability of Hb Madrid is, first, that insertion of the proline at position G17 will disrupt part of the [illegible] helix, also, and perhaps more important, alanine G17, which is common to the α-, β-, γ- and δ-chains, is one of the amino acid residues at the $\alpha 1\beta 1$ contact [22]. The side chain of proline, which consists of three methylene groups of the pyrrolidine ring, is much more bulky than the methyl group side chain of alanine; introduction of the proline residue at G17 could force apart the α- and β-chains at the $\alpha 1\beta 1$ interface. Thus the stability of this contact would be broken, leading to dissociation into unstable monomers.

Haemoglobin Madrid is the eighth example of an unstable haemoglobin in which a neutral amino acid has been replaced by a residue of proline (table III). All these variants, with one exception, have resulted in a moderate to extremely severe haemolysis in affected heterozygotes. The exception, Hb Duarte [2], is interesting. The propositus was doubly heterozygous for Hb Duarte and β-thalassaemia, and therefore had no Hb A; he suffered from a moderately severe anaemia. However, the parent, who was heterozygous for Hb Duarte and Hb A, suffered only a mild degree of haemolysis.

In UHHAs, the severity of the disorder is determined by both the type and site of mutation. For example, replacement of the bulky hydrophobic phenylalanine β42 (CD1) by the small, hydrophilic serine in Hb Hammersmith [11] causes a more serious disease than that caused by the substitution, in Hb Bucuresti (Louisville) [3] of that phenylalanine by leucine, which is also hydrophobic and only somewhat smaller than phenylalanine. On the other hand, the same mutation as seen in Hb Hammersmith (Phe → Ser), at another site – in Hb Christchurch (β71 E15) [6] – is also associated with a more benign condition than Hb Hammersmith disease; the reason for this is that residue CD1 is of especial importance for stability of the haem pocket. Indeed, of all the known normal haemoglobin and myoglobin chains, CD1 is one of the very few residues which is completely invariant.

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Acta Haemat. 52: 61-64 (1974)

Trisomy D in Bone Marrow Cells in a Patient with Chronic Myelogenous Leukemia[1]

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Abstract. A female patient with chronic myelogenous leukemia was found to have 47,XX,+D in 100% of her bone marrow cells. There was no Ph[1] chromosome. Fluorescent staining showed that the extra D group chromosome was most likely a No. 13 chromosome. Both lymphocyte and skin fibroblast cultures revealed a normal female karyotype which indicated that the abnormal karyotype was not constitutional but an aberration occurring only in the leukemic bone marrow cells. To our knowledge, such an aberration has not been reported previously as an isolated abnormality present in leukemic marrow cells.

Key Words
Chromosome aberrations
Chronic myelogenous leukemia
Karyotype
Leukemic marrow cells
Trisomy D

The presence of the Ph[1] (22q-) chromosome in patients with chronic myelogenous leukemia (CML) has been well documented. It is the only unique chromosomal abnormality found consistently in human neoplastic diseases. A minor proportion of the patients with CML has been found to be Ph[1] negative; these patients are likely to have atypical forms of the disease and tend to have a poorer prognosis [5, 8]. We wish to report a different type of chromosomal aberration, 47,XX,+D, found in the bone marrow cells of a patient with atypical CML. To our knowledge, such an aberration has not been reported previously as an isolated abnormality present in leukemic cells.

[1] This work was supported in part by US Public Health Service grant HD-02552, CA 10515, and Clinical Genetic Center grant GM 19443.

Case Report

The patient a 68 year old black female was admitted to Mount Sinai Hospital in March 1972 with a presumptive diagnosis of CML. The chief complaints were intermittent sharp pain in the thighs and left buttock for 3 weeks Workup showed a mild anemia thrombocytosis, and leukocytosis with a shift to the left (see laboratory data below)

Past history The patient had pleurisy at the age of 26 In December 1970 she had tuberculosis of supraclavicular nodes and was treated with antituberculosis medication The patient had a normal menstrual history, and she gave birth to 10 normal children before reaching age 40 There were no known congenital abnormalities in the family

Physical examination A slim black woman who had normal secondary sex characteristics She had rather pale conjunctivae and several palpable axillary lymph nodes There was no hepatosplenomegaly She did not have any of the stigmata associated with congenital trisomy 13

Dermatoglyphics showed slightly distally located palmar triradii (t') The finger tip patterns revealed 6 ulnar loops and 4 arches (on the right 2nd and 3rd and left 3rd and 5th fingers)

She expired 4 months later after a course marked by fluctuation of leukocyte and platelet levels intermittent fevers of unknown origin and terminal pneumonia endocarditis and pericarditis Slight hepatosplenomegaly and increasing anemia requiring transfusions developed terminally No antileukemic therapy was given because her other medical problems were more troublesome clinically and not necessarily related to leukemia and because the diagnosis of leukemia was not certain until the autopsy showed infiltrates of immature leukemic cells in the liver spleen lymph nodes, kidneys and adrenals No active tuberculosis was found The pathologic diagnosis was myeloid leukemia

Laboratory Data On admission Hb was 10 2 g%, platelets 729 000 mm³, and WBC 31 200 mm³ with 48% segmented neutrophils 19% bands 3% metamyelocytes 7% myelocytes 2% promyelocytes 1% eosinophils 1% basophils 7% monocytes and 12% lymphocytes A bone marrow aspirate was hypercellular with increased numbers of megakaryocytes and a myeloid erythroid ratio of 5 3/1 The granulopoiesis had a slight shift to the left with 6% promyelocytes and 9% myeloblasts Subsequent blood counts showed some variation in the WBC/mm³ occasionally myeloblasts were seen. Bone marrow aspirates did not change significantly The sedimentation rate was 45 mm/h Leukocyte alkaline phosphatase was less than 20 on one occasion but normal on others She did not have a Philadelphia chromosome (see below)

Cytogenetic findings Direct bone marrow preparation without stimulation by phytohemagglutinin for chromosomal analysis showed 47 chromosomes with an extra D group chromosome in all 30 metaphases examined (fig 1) In order to exclude the possibility that the abnormal karyotype might be constitutional a peripheral leukocyte culture stimulated by phytohemagglutinin and a skin fibroblast culture from a skin biopsy were also studied Both of these showed a normal female chromosomal constitution 46 XX Ten cells were examined from the lymphocyte culture and 25 cells from the skin fibroblast culture A buccal smear

Fig 1 Partial karyotype of D group chromosomes from a bone marrow metaphase cell showing an extra D chromosome

showed one normal sized X chromatin body in 30% of the nuclei examined, thus further confirming the XX constitution of the patient

For further identification of the extra D group chromosome in the bone marrow cells, fluorescent staining with quinacrine dihydrochloride was applied [1] The fluorescent staining showed 3 D group chromosomes with bright fluorescence especially on the distal two thirds of the long arm 2 D group chromosomes with fluorescence concentrated at the proximal half of the long arm and 2 D group chromosomes with relatively less fluorescence Thus, the fluorescent patterns of the D group chromosomes were consistent with an extra No 13 chromosome [2]

Discussion

The finding of 47 XX +D in all bone marrow cells of this patient but not in her lymphocytes and skin fibroblasts indicates that the abnormal karyotype is not constitutional but is an aberration occurring only in the bone marrow cells The clinical features and the laboratory data of this patient were suggestive of CML although they were not typical No Ph[1] chromosome was found Fluorescent staining of the bone marrow metaphase chromosome showed that the extra D group chromosome is most likely a No 13 chromosome

To our knowledge trisomy D as an isolated chromosomal aberration has not been previously found in leukemic cells Of 103 patients with acute myelogenous leukemia in one study, 7 had trisomy D in their marrow, but all had various other chromosomal aberrations as well [8] Of 179 patients with CML 158 were Ph[1] positive, and 3 of these had trisomy D in the marrow but again with other chromosomal abnormalities [7] Various different types of chromosomal aberrations have been found in the blastic crisis of patients with CML, but generally these have associated with one or more Ph[1] chromosomes [6] Five of 8 patients in blastic

crisis of CML, all with Ph[1] chromosome, had extra D chromosomes in their marrow, but each had other chromosomal aberrations as well [4].

In general, there is a chromosomal instability in patients with acute leukemia or blastic crisis of CML, and the finding of trisomy D associated with other chromosomal aberrations in some of the leukemic cells appears to be nonspecific. In contrast, the finding of an isolated chromosomal abnormality in 100% of the marrow cells of this patient suggests that a single abnormal leukemic stem cell gave rise to myelocytic, erythrocytic, and possibly megakaryocytic precursor cells. In 1967, Fialkow *et al.* [3] demonstrated the clonal origin of CML in man. It is conceivable that malignancy in general arises as a consequence of a chromosomal event or mutation in single stem cell.

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Acta Haemat. 52: 65–69 (1974)

Serum Concentrations of Haptoglobin and Hemopexin in Favism and Thalassemia

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Abstract. The serum levels of haptoglobin (Hp) and hemopexin (Hx) have been investigated in favism and thalassemia. In 25 children with favism, Hp was absent in 22 and both Hp and Hx in 7 cases. In 45 children with Cooley's anemia, Hp was absent in 33 and Hx in 37 cases. In 35 adult carriers of thalassemia minor, the findings were similar to those observed in healthy controls. The absence of Hx in spite of moderate hemolysis in most cases of Cooley's anemia is emphasized.

Key Words
Erythrocyte G-6-PD deficiency
Favism
Haptoglobin
Hemolytic anemias
Hemopexin
Thalassemia

In some hemolytic diseases, haptoglobin (Hp) and hemopexin (Hx) levels may be low, the former also reacting to any slight increase in red cell turnover, the latter being more or less affected in cases of marked hemolysis. The observations of SEARS [9, 10] have indeed shown that Hx only drops when the hemoglobin (Hb) binding capacity (HbBC), which is dependent on Hp, is exhausted. In this paper, the validity of such an assumption is examined in acute as well as in chronic hemolytic diseases. Therefore, favism and thalassemia were investigated because both are very common in Sardinia, and thus serial researches could easily be carried out in a large group of cases. In the literature, where the two above-mentioned diseases are concerned, data on the relationship between Hp and Hx levels are very limited in number.

Materials and Methods

Subjects studied. 25 normal healthy children aged between 3 and 12 years (mean age 6 years) in whom hematological traits were previously excluded

25 children aged between 3 and 11 years (mean age 5 years) with erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) deficiency were admitted to our hospital in May 1973 because a severe hemolytic crisis accompanied by hemoglobinuria had occurred in all but 3 cases (intermediate females) after ingestion of fresh fava beans. This group was randomly selected out of a total of 42 cases observed during this month. In these cases the onset of hemolysis was from 1 to 4 days before admission.

45 children with Cooley's anemia (21 males and 24 females) aged between 2.5 and 10 years (mean age 6 years). These children were studied during one of their periodic admissions to our hospital for routine transfusions from April 1 to May 25, 1973. Their mean Hb level on admission was 6.6 g% (range 3.2–8.0 g%). They were not splenectomized and in all cases a course of desferrioxamine treatment (70 mg/kg/day during 10 days after transfusion) was followed for 2 weeks after the last admission. Except for 2 cases which received no transfusion for as long as 2 months, the remaining were transfused (using erythrocyte concentrates) every 25–35 days according to our transfusional program.

25 adult carriers of the thalassemia trait (11 males and 14 females) aged between 22 and 40 years (mean age 34 years) were randomly selected from fathers and mothers of children with Cooley's anemia.

Methods. Hb was determined by Drabkin's method, erythrocyte G-6-PD by the method of KIRKMAN and RILEY [4]. Hp and Hx were measured in the serum by the single radial immunodiffusion technique as described by MANCINI *et al.* [8] using M-partigen's immunodiffusion plates (Behringwerke). The Hp typifying was previously performed according to SMITHIES [11] on starch gel and the genetic Hp type has been taken into consideration for quantitative determination.

Results

A comparison between the mean Hp and Hx values in the 4 groups is given in table I.

In favism Hp was absent in 22 and both Hp and Hx in 7 out of 25 cases, all characterized by hemoglobinuria and a severe reduction in Hb levels. In the remaining patients (3 girls whose erythrocyte G-6-PD activity was 50% in comparison with the normal range) hemoglobinuria did not occur.

In thalassemia major Hp was absent in 13 and Hx in 37 out of 45 cases, and in the cases in which Hp and/or Hx could be detected the mean values were significantly lower than in the controls (table I). The distribution of the values Hx 0 is approximately the same in relation to age and number of transfusion (table II).

In thalassemia minor there was no significant difference in Hp and Hx concentrations when compared with the controls (table I).

Table I Hp and Hx serum concentrations (mg/100 ml)

	Number of cases	Hp mean ± SD	Hx mean ± SD
Controls	25	219 04 ± 96 [illegible]	95 76 ± 31 09
Favism	7	0	0
	15	0	49,73 ± 33 [illegible]
	3	4, 22, 80[1]	26, 45, 80[1]
Thalassemia major	9	0	0
	4	0	8, 8, 12, 26[1]
	4	20, 100, 156, 166[1]	8, 16, 16, 49[1]
	28	111 42 ± 84 09	0
Thalassemia minor	25	186 08 ± 104 [illegible]	101 12 ± 31 76

[1] Values are shown individually because of the small number of examples

Table II Distribution of thalassemia major cases showing Hx level = 0 in relation to age and number of transfusions

Age groups	Number of cases	Number of transfusions (range)	Hx = 0 (number of cases)	%
Less than 3 years	6	10–19	5	83 33
3–6 years	15	22–63	12	80 00
Above 6 years	24	58–130	20	83 33

Discussion

The average mean values of the Hp and Hx plasma levels in thalassemia minor (table I) are in the same order of magnitude as those found in the controls YAMAK and OZSOYLU [5], however, have found that Hp, determined by electrophoretic methods, may be absent in some instances As far as we know, there are no findings obtained by immunodiffusion techniques comparable to our data In any case, it seems that hemolysis, when present, is so moderate in thalassemia minor, that it cannot significantly affect the Hp turnover

The fall in Hp serum level in thalassemia major [1, 3, 5–7, 9, 10] and favism [1, 2] is consistent with the hemolytic state and it could be predicted that Hp depletion in the latter should be more pronounced. There is indeed no doubt that the hyperacute hemolysis which occurs in favism represents a singular condition which during the course of 24–36 h affects about 75% or more of the total erythrocyte mass. Hb released in the blood stream depletes the binding capacity of Hp and hemoglobinuria appears. It is noteworthy that in the three cases of partial G-6-PD deficiency hemoglobinuria did not occur. In these cases, however, hemolysis was severe and could be approximately estimated (on the basis of residual Hb) at about 40%.

The relationship between the fall in Hp and Hx in favism is very clear. Hp depletion in all but the three intermediate females, in the remaining 22 a reduction in Hx to half the normal mean value in 15 and its disappearance in seven. SEARS' [10] theory that Hx depletion only takes place when Hp is depleted is confirmed by these findings.

As far as the changes of Hp and Hx in chronic hemolytic disorders are concerned, extensive studies have not yet been carried out. In a report on a few cases of thalassemia major-sickle cell anemia and sickle cell-HbC disease, MULLER-EBERHARD *et al.* [6] and MULLER-EBERHARD [7] observed that Hp and Hx were both equally depleted. In our material, such a finding was present in 9 out of 45 cases. In the remainder, Hx was absent in 28 in spite of a mean Hp concentration of 111.42, i.e. about half the mean value found in the controls. In four cases only Hp was absent and residual Hx could be detected, in four patients both Hp and Hx were present at low levels. These data suggest that the relationship between Hp and Hx in thalassemia major is more complex than would appear from isolated determinations. According to our results it seems likely that Hx depletion is the main feature present in 80% of cases, irrespective of age group and number of transfusions (table II). A fall in Hp evidenced by many other authors, too, is a constant finding less pronounced in thalassemia major than in favism but consistent with relatively moderate hemolysis.

It has been stated that Hx depletion is dependent on the high concentration of free heme which complexes with Hx and albumin [6]. In excessive hemolysis the heme concentration increases as a consequence of the dissociation of part of the Hb circulating in the plasma into its components heme and globin. In these circumstances, evidently, HbBC is saturated and Hx depleted. However, the disappearance of Hx only

which we found in most cases of thalassemia major, cannot be explained by such a pathway and rather suggests that Hx depletion is not caused by the hemolytic component of the disease. We did not determine the heme concentration in our cases as MULLER-EBERHARD [7] did, but his results, though limited to a few cases, draw attention to the problem of the increased circulation of heme in thalassemia major which could explain this Hx depletion.

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Acta Haemat 52 70–76 (1974)

Experimental Study on Haematotoxic and Leukaemogenic Effects of Trichlorophone and Dimethoate

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Abstract The haematological effects of the economically important alkylphosphates, trichlorophone and dimethoate, were tested experimentally on rats A pronounced haematotoxic action of these pesticidal organophosphate compounds was found including severe hyperplasia of the haematopoietic parenchyma of bone marrow and extra-osseous myeloid metaplasia particularly in liver and spleen The interpretation of the results in terms of a myeloproliferation (panmyelosis) and myeloid leukosis and their practical implications are discussed

Key Words
Dimethoate
Haematotoxicity
Myeloid metaplasia
Myeloproliferative syndromes
Pesticides
Trichlorophone

Pesticidal organic phosphate compounds have assumed leading positions among synthetic insecticides as a genuine alternative to DDT and other insecticides on halogen hydrocarbon basis with their lasting environmental contamination due to high persistence However, strongly alkylating properties and associated cytotoxic effects were claimed for some of them in more recent reports Quite a number of alkylants have even been used as cytostatics for their cytotoxic action

Only some scarce information so far has become available regarding potential bone marrow damage by pesticides and insecticides on the basis of organophosphorous compounds, while much more experience has been recorded from halogen hydrocarbon insecticides such as HCH and DDT Yet, it should be borne in mind that in their semi-annual tabulation for 1965, more than 20 pesticides were associated to the genesis of

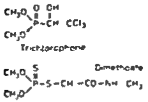

Fig 1 Trichlorophone dimethoate

blood damage by the Panel on Hematology of the Registry on Adverse Reactions in the Council on Drugs of the American Medical Association [1] with the organophosphorous insecticide parathion being placed next to chlordane and γ-HCH as a compound of established haematotoxicity *In 1972* SANDIFER *et al* *[3] published 4 year observations of persons* occupationally exposed to pesticides in South Carolina and reported significant leucocytosis for those groups of workers who had been exposed predominantly to organophosphorous compounds

However, long term animal experiments of coherent information on the haematotoxicity of organophosphorous compounds still are lacking Therefore the authors have undertaken experimental studies using rats, with the purpose to test the effects in terms of the haematotoxicity of trichlorophone and dimethoate (fig 1) alkyl phosphates of major economic importance

Material and Methods

Tested substances Aqueous solutions of commercial preparations of trichlorophone and dimethoate

Test animals Wistar rats aged 10 weeks

Application Twice per week until spontaneous death trichlorophone 15 mg kg by tube or intramuscular injection 40 rats each dimethoate 5 10 and 15 mg kg by tube or 15 mg kg i m 40 rats each

Histological evaluation was restricted to animals which had been under experimentation more than 3 months with no post mortal change being exhibited by their organs Physiological sodium chloride solution was administered to control animals by tube intramuscularly or percutaneously

The informative value of post mortal cytological bone marrow examination on the basis of smear or crushed preparations is limited and exclusive histological examination of spleen lymph nodes and liver in experimental animals will be sufficient for safe haematological diagnosis only in rare cases Therefore the follow

Table I

Single dosis, mg/kg	Application	Average survival days	Number of animals		Haematological findings	
			at beginning	for evaluation	myeloproliferation, %	extra-osseous myeloid metaplasia, %
Trichlorophone						
15	oral	654	40	28	47	34
15	i m	565	40	27		
Dimethoate						
5	oral	518	40	26		
15	oral	511	40	25	57 6	59
30	oral	627	40	20		
15	i m	570	40	30		

ing tests were made in a very broad approach (1) leucocyte count, (2) blood smear and leucocyte differential count, (3) cytological evaluation of femoral bone marrow smears, (4) histological examination of bone marrow in femoral and vertebral bodies, (5) histological examination of all organs, with particular emphasis on spleen and liver Strong emphasis was laid on histological bone marrow examination, as some experience had been recorded earlier along this line from man and rats [2, 4, 5]

The bone marrow of some animals could not be tested for external reasons Repeated blood tests were applied to some of the animals, using sparing and non interfering puncture of the retrobulbar venous plexus Standard values for reference were obtained from controls of the same species

Results

The most important haematological results included pronounced hyperplasia of the haematopoietic parenchyma in the bone marrow and, sometimes strong extra-osseous myeloid metaplasia which occurred mainly in liver and spleen, but also in other organs, for example the lungs (table I)

Fig 2 Hyperplasia of bone marrow with preferential involvement of granulocytopoiesis after dimethoate Vertebral body

Fig 3 Hyperplasia of bone marrow with somewhat equal involvement of all 3 systems (panmyelosis or myeloproliferation) after trichlorophone Vertebral body


2
3

Table II Leucocyte counts after trichlorophone and dimethoate

	% of tested animals (n = 80)	Leucocytes	
		mean value	standard deviation
Trichlorophone	23	27 407	10 706
Dimethoate	36	27 760	10 100
Control group		12 212	2 531

Hyperplasia in the bone marrow occurred at different levels more or less in all 3 haematopoietic cell systems however with preferential involvement of granulocytopoiesis

Leucocytosis of varying intensity in the peripheral blood was an additional finding which affected mainly the granulocytes (table II) The highest values exceeded 50 000/µl For animals on which histological examination was also performed this phenomenon was in agreement with the above findings Some examples of findings in bone marrow spleen and liver may be seen from the figures 2–4

Discussion

The changes in the haematopoietic systems of rats may be interpreted as direct haematological effects of trichlorophone and dimethoate They should be associated with the myeloproliferative syndrome of which different morphological manifestations and haematological courses are known depending on the given phase of the disease Exclusive morphological differentiation of extra osseous myeloid metaplasia with concomitant myeloproliferation and straightforward interpretation as leucosis may be problematic The animals exhibited hyperplasia of all systems although granulocytopoiesis was predominant in most cases which may in fact be myeloid leucoses

No matter the decision taken with regard to classification of the haematological changes the results of the study justify the demand that haematological parameters should be included in regular routine checks of individuals handling such alkyl phosphates both in manufacturing industries and in agriculture The findings also seem to be important to classify patients with a history of exposure to such compounds

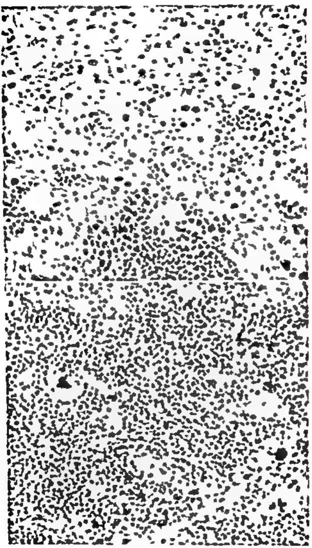

Fig 4 Extra-osseous myeloid metaplasia *a* in the liver after dimethoate, *b* in the spleen after trichlorophone

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Acta Haemat. 52: 77–82 (1974)

Study of Post-Splenectomy Thrombocytopoiesis with ^{75}Se-Methionine in Mice

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Abstract. The number of circulating platelets gradually increases in splenectomized mice, exceeding the starting level of 50% during the second week. Nearly identical amounts of ^{75}Se-methionine given at different times after splenectomy, or in the control group after sham-operation, appear in the circulating platelets of both experimental groups of mice. It is unlikely that increased platelet production could play a decisive role in the development of post-splenectomy thrombocytosis.

Key Words
Platelet kinetics
Post-splenectomy thrombocytosis
^{75}Seleno-methionine incorporation
Spleen effect on bone marrow
Thrombocytopoiesis

In cases of splenomegaly of various origin thrombocytopenia frequently disappears after splenectomy. Removal of the normal spleen is also followed by thrombocytosis. According to an early view [10] the spleen eliminates the aged or damaged platelets. Another view [9] attached importance to the humoral inhibitory substance of the spleen in the development of 'hypersplenism'. Later, several workers emphasized the platelet-storing (pool) role of the spleen [1, 7, 8, 17].

After splenectomy the level of circulating thrombocytes increases in experimental animals, thus also in mice. The mechanism of this thrombocytosis is still not clear in its details. Three factors may be involved: (a) thrombocytopoiesis may be increased, i.e. more new cells are formed, (b) the lifetime of circulating platelets may become longer, and (c) the absence of splenic platelet-pooling may be the cause.

Earlier, we tried to approach the problem by studying the megakaryocyte system of the bone marrow [15]. Our results did not prove increased thrombocyte production. In our present work we studied the thrombocytopoiesis in splenectomized mice using radioisotope technique.

Materials and Methods

Inbred male and female 3 to 4 month-old BALB c mice weighing 35 g on average and kept on standard diet were used Blood samples were obtained from the tail vein and the platelet count was determined by phase contrast microscopy [12] To minimize blood loss only two platelet counts were done on each animal The mean platelet count of 40 untreated mice was $1\ 115 \pm 0\ 105 \times 10^{6}$ (1 SD)

The isotope used ^{75}Se methionine (^{75}Se Met) is the γ radiating radioactive analogue of natural sulphur methionine (Radiochemical Centre, Amersham England) Its specific activity is 5 mCi/mg It was dissolved in physiological saline, and 2 μCi in a volume of 0 2 ml was given intraperitoneally to each animal Incorporation of ^{75}Se Met into platelet precursors was studied by the method described by PENINGTON [16] and slightly modified by us [18] The main steps of the method are as follows 0.5 ml of blood obtained from axillary blood vessels was added to 7.5 ml of 5% EDTA Na_2 (pH 6 5), then platelet rich plasma was obtained by differential centrifugation a washed platelet concentrate was made from this and resuspended in EDTA solution Radioactivity and the activity of standard isotope solution (containing 1/100 of the total given dose) were measured in a well scintillation γ-detector (Packard Tri Carb-Liquid scintillation spectrometer) for 300 sec Radioactivity of the circulating platelet mass was determined by the formula the count obtained from platelet samples×blood volume (ml/g)×body weight (g)×2 Multiplication by 2 is necessary because the samples contain the radioactivity of platelets of 0.5 ml blood The results are expressed in percentage of administered isotope dose which is calculated by dividing the count indicating the radioactivity of the circulating platelet mass by the standard count and multiplying by 100 ($\% \times 10^{-2}$)

The blood volume of mice was determined as 0 084 ml/g using the radiochromium method

Radioactivity of platelets was measured 48 h after injection of ^{75}Se Met The average of the isotope incorporation of 12 untreated mice was $4\ 43\% \times 10^{-2} \pm 1.27\% \times 10^{-2}$ (1 SD)

The untreated (control) group consisted of 12, the splenectomized and the sham operated groups of 50 mice each at each measurement of radioactivity the values of 10 mice were determined

The figures show the average values and the standard error (SE) of the platelet counts the ^{75}Se Met content of the circulating platelet mass in percent (average and SE) the average of the platelet counts of untreated mice the average isotope content of the platelets their standard deviation (SD) and SE

Results

Following splenectomy the platelet count gradually increases from a value around 1 1 M at the start to 1 6 M on the fourth day and to a value around 1 75 M on the ninth day Thus, the rise of the platelet level is about 50% After the ninth day there is a slow decrease and values near to the initial values can be seen between the third and the fourth weeks

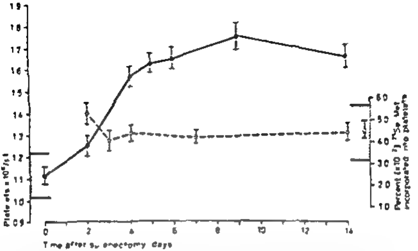

Fig 1 Platelet counts and ^{75}Se-Met incorporation after splenectomy On the figures the magnitude of incorporation is represented at the time of isotope administration, while the radioactivity incorporated into megakaryocytes and subsequently carried in the blood by the platelets was really measured only 48 h later

Incorporation of ^{75}Se-Met into the platelet precursors 48 h after splenectomy is moderately increased (5 27%× 10^{2}), on the other days of checking it is similar to the values of the non-splenectomized group (fig 1) It should be emphasized that on the figures the magnitude of incorporation is represented at the time of isotope administration, while the radioactivity incorporated into megakaryocytes and subsequently carried in the blood by the platelets was really measured only 48 h later

A moderate rise of the platelet level (1 43 M) with a maximum on the fourth day was observed after the sham-operation (incision over the spleen) The level decreased within days and was normal by the end of the second week. The incorporation of ^{75}Se-Met into the platelet precursors 48 h after the sham-operation is similar to the splenectomized group, near the upper limit of the normal value (5 21%× 10^{2}) The subsequent values are near to the average value of the untreated group (fig 2) That means that differences were found in the degree and duration of the postoperative thrombocytosis between the splenectomized and the sham-operated groups but that there was no appreciable difference in the ^{75}Se-Met activity of the circulating platelet mass

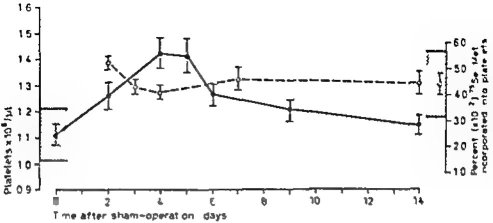

Fig. 2 Platelet counts and ⁷⁵Se Met incorporation after sham operation

Discussion

Study of the ⁷⁵Se-Met incorporation seems to be suitable for quantitation of the bone marrow megakaryocyte mass and of platelet production ⁷⁵Se-Met incorporates into proteins in the same way as the sulphur-containing natural methionine Incorporation has been found to vary with changes in the rate of platelet production It appears to label newly produced platelets during their formation in megakaryocytes and provides a method by which thrombocytopoiesis may be studied Direct labeling of platelets in the circulation does not occur [11, 16] Experimentally influenced thrombopoiesis stimulated by anti-platelet serum [18] or suppressed by platelet transfusion [20] can be well followed using this method It can be expected that measuring of the radioactivity of the circulating platelets after isotope administration will inform us about the bone marrow megakaryocyte mass and the rate of thrombopoiesis also in the post-splenectomy period

No difference was found in the incorporation between the splenectomized and the sham-operated groups The initial moderate increase in both groups may be due to the operation itself [5] and may explain the increase of the circulating platelets after the sham-operation and partly after splenectomy At the time of more intense and more lasting post splenectomy thrombocytosis a further increase in incorporation indicating augmented production was not observed up to 2 weeks after splenectomy

In our earlier investigations the bone marrow megakaryocyte content was found to be unchanged after splenectomy and even slightly decreased on the fifth and eighth post-operative day [15]. ROLOVIC and BALDINI [19] using autoradiography found the rate of megakaryocyte maturation unchanged in rats after splenectomy. We did not examine the problem, but according to others the lifespan of the platelets does not increase after splenectomy [3, 6, 13, 17].

The spleen as a platelet store may play an important part in the distribution of the platelet mass in the organism. In humans one third of the platelets can be found in the spleen [1]. Similar data were reported by PENNY *et al.* [17]. According to ASTER [2] 10–15% of all platelets of the rat are in the spleen. We have no numerical data as regards the mouse. According to ASTER [4] loss of the spleen as a store does not satisfactorily explain post-splenectomy thrombocytosis, at least in humans, increased production must also be supposed. Elimination of the longdisputed inhibitor substance of the spleen might be the cause of the increased platelet production. Several workers [14, 21] could prevent post splenectomy thrombocytosis by reimplantation of a small portion of the spleen. We observed similar thrombocytosis in mice after reimplantation of 10–20% of the spleen as in the splenectomized controls (unpublished data).

Experimental data available till now do not explain clearly the mechanism of post-splenectomy thrombocytosis. It seems that increased thrombocytopoiesis in the period following splenectomy cannot be demonstrated even by isotope technique. Here again the importance of the elimination of the splenic storage function can be emphasized. It is not clear, however, why the radioactivity of the circulating platelets does not increase following splenectomy even if the rate of platelet production is unchanged, for we can suppose that labelled cells would also get into the splenic pool. If, in the splenectomized group, the larger mass of circulating platelets has the same radioactivity as the smaller platelet mass of the sham-operated group, decrease of platelet volume can be expected after splenectomy. The investigation of this problem is the subject of our present studies.

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Acta Haemat. 52: 83-94 (1974)

Biochemical and Morphological Observations on Lymphocytes in Continuous Cell Culture from a Patient with Chronic Lymphocytic Leukemia[1]

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Abstract. A continuous cell culture was established from unstimulated peripheral blood lymphocytes of a patient with chronic lymphocytic leukemia. The cells grew singly and in free floating aggregates, and the population was mixed and contained lymphoid, plasmacytoid, reticulum cells, and blast cells. HL-A typing of the established cell line was identical to the preculture cells, and remained unaltered. Electron microscopy demonstrated HLV particles. Although aneuploidy was present, the predominant chromosomal mode was 46, male. Glycolytic enzyme patterns showed predominance of the enzymes utilized in the anaerobic pathway. The cell generation time was found to be 30-50 h.

Key Words
Electron microscopy
Leukemia cells
Lymphocyte culture
Lymphocyte enzymes
Lymphocytic leukemia

Long term cell lines in culture have been established from peripheral blood lymphocytes of normal individuals as well as from patients with acute leukemia and chronic myeloid leukemia. Cell lines have also been established from lymph node specimens of patients with lymphosarcoma and Burkitt's lymphoma [1, 10, 12, 22, 25]. Among the nonneoplastic disorders, peripheral blood lymphocyte cell lines have been easily established from cases of infectious mononucleosis, herpes simplex, herpes zoster, mumps and measles [14, 15, 16]. These disorders share a viral etiology and the finding of 'atypical' lymphocytes.

[1] Supported by grants from United Leukemia Inc., the National Leukemia Association and Chemotherapy Foundation of New York, Inc.

The difficulty of establishing lines from normal individuals without using large amounts of blood can be obviated by adding mitogenic substances to the culture. Establishment of a long term cell culture from the peripheral blood lymphocytes of a patient with chronic lymphocytic leukemia would not be likely, since the cell population of this disorder is poorly responsive to mitogenic stimulation [24, 26]. This report concerns successful establishment of such a culture without mitogenic stimulation.

Case Report

M. G., a 46-year old white male, was found to have chronic lymphocytic leukemia during a routine physical and laboratory evaluation in April 1968. He was asymptomatic and only a minor degree of splenomegaly was found on physical examination. His hemoglobin was 15.0 g%, and the white blood cell count was 46 000/mm³, of which 86% were small lymphocytes. The bone marrow was hypercellular and 64% of the nucleated cells were lymphocytes.

The patient was observed without specific therapy. In October 1969, following a severe pharyngitis and an associated vesicular eruption on the face, thought to be herpes simplex, the white blood cell count rose from 50 000 to 200 000/mm³ and generalized lymphadenopathy became apparent. Peripheral blood was obtained for *in vitro* culture at this time (vide infra). The patient was then treated with Chlorambucil with good response. His disease regressed and he has continued to remain well until the present time (January, 1974).

Methods

10 ml peripheral blood was drawn into a heparinized syringe and allowed to sediment at 37 °C. The white cells were removed, washed once with 10 ml RPMI 1640 or TC199 media, and parallel cultures set up in 10 ml of either one of those media supplemented with 20% fetal calf serum (Grand Island Biological Company), penicillin, streptomycin and L-glutamine. Final cell concentrations were 10⁶ cells/ml. 0.1 ml of phytohemagglutinin (PHA) (Burroughs Wellcome) was added to one third of the flasks, pokeweed mitogen (PWM) (GIBCO) was added to a second third, and no mitogen to the remaining flasks. Media were changed twice weekly for the first 3 weeks and weekly thereafter.

Trypan blue dye exclusion was used to determine cell viability. Morphologic observations were made on films stained with Wright-Giemsa. Euchrysine supravital staining was done by the method of BLUME *et al.* [2]. Electron Microscopy was performed using a Siemens 101 electron microscope and previously described methods [8, 9]. Karyotypes of both the patient's peripheral blood and of the cells in culture were made periodically using standard methods. The activity of the enzymes hexokinase, glucose-6-phosphate dehydrogenase, aldolase, fructose 6-phosphate kinase and

pyruvate kinase were determined using previously described methods [4, 5, 18, 19, 27]. HL-A typing of the cells before and during culture was done at the New York Blood Center by Dr. M. Fotino [13]. The cell doubling time was measured at 31 and at 69 weeks of culture. At each observation period, cell sub-culture innoculae were counted in duplicate at the time of subculture (0.8–1.4 × 10^6 cells/ml) and the new culture population counted again after 72 h of incubation.

Tritiated thymidine (^{3}HTdR) (New England Nuclear Corporation, specific activity 6.7 Ci/mM) labelling studies were performed on these cells during the 69th week of culture. After 2 h of incubation the cells are washed three times, resuspended in 19 ml of medium containing 20% fetal calf serum. At intervals of 0, 2, 4, 24 and 48 h, an aliquot of cells was removed. The cells were counted, viability determined, aliquots were prepared, and liquid scintillation was done in duplicate. Perchloric acid, hydrogen peroxide extraction, and Unogel scintillation fluid (Schwarz/Mann) were used.

Results

For the first 4 weeks 60–80% of the cells in all flasks remained viable and demonstrated limited growth. After 5 weeks in culture, whereas the cells in all of the flasks containing TC199, PHA, or PWM were mostly dead, those cultures containing RPMI 1640 *without* mitogen had an increased media requirement. An exponential growth curve was observed when subcultures of 0.8–1.4 × 10^6 cells/ml were innoculated into fresh media at 35 weeks and again at 69 weeks of culture. The cell doubling times at both periods were similar, ranged from 30 to 50 h, and slowing of growth occurred after 72 h. After flash labelling with ^{3}HTdR, there was no change in cell uptake/10^6 cells, and no increase in cell number up to 24 h (table I). At 48 h a more than 2.5 fold increase in cell number and a simultaneous fall in uptake per 10^6 to 23–43% of the zero hour values occurred ($p < 0.01$).

Morphologic Observations

Single or clumped free floating cells were seen (fig. 1) by inversion microscopy. Wright-Giemsa stained smears revealed a mixed population of cells from 10 to 30 μm diameter. The nuclei were finely granular with prominent nucleoli and the cytoplasm basophilic and often vacuolated. Euchrysine supra-vital staining of the cells in culture demonstrated large numbers of cytoplasmic organelles with a variable staining intensity. Histochemical staining by the periodic acid Schiff (PAS) method indicated an increase in the cell glycogen content when these were compared to stained pre-culture cells.

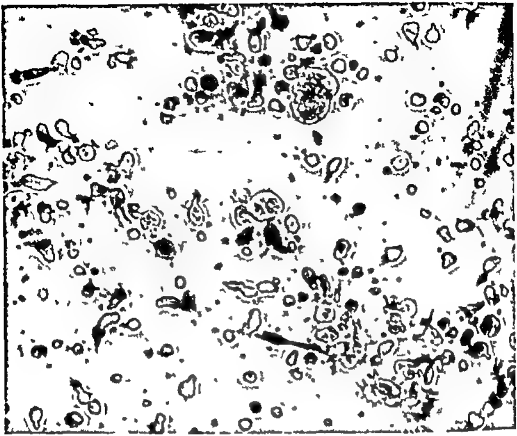


Fig 1 Cells in suspension culture rosette forms pseudopod formation and mitotic figure × 200

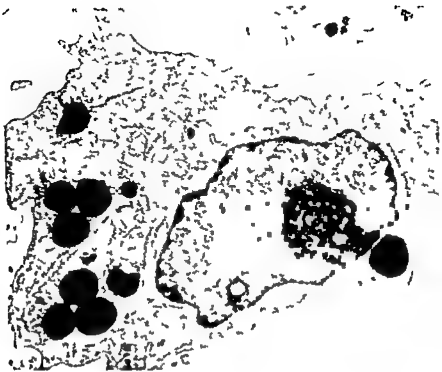
Table 1 Chronic lymphocytic leukemia cells in continuous culture Proliferative activity of ^{3}H thymidine labelled cells

Time in culture, h[1]	Viable cells (dye exclusion) %	Leukocyte count[2] μl^3	Tritium radioactivity cpm/10^6 cells
0	97	2 600	6 200
		3 100	4 800
2	95	3 600	4 200
		4 300	3,300
4[3]	85	3 100	5 800
24	90	2 900	6 300
		3 300	4 000
48	95	6 800	2,700
		8,400	1,100

[1] After ^{3}H TdR flash labelling

[2] The two sets of data represent observations from two flasks run as duplicate

[3] Only one flask was sampled at 4 h



F g 2 Lymphoblastoid cell conta n ng many polysomes well-developed RER large electron dense l p d bod es × 9,375

On electron microscop c examination the predom nant cell populat on had cytoarch tectural characteristics of lymphoid cells The nuclei were irregular in shape and had prominent nucleoli (fig 2) Occas onal plasma cytoid cells with widely d lated and prominent rough surface endoplasmic reticulum were present Occasional HLV particles were seen (fig 3)

Chromosome Studies

The original short term culture showed a normal male d ploid kary otype More than 90% of metaphases in samples of cultures taken after 7 15 30 54 and 75 weeks had the normal male diploid complement How ever abnormal ties such as aneuploidy of 45 chromosomes tetraploidy and endoredupl cation were also observed HL-A typing of the cells both

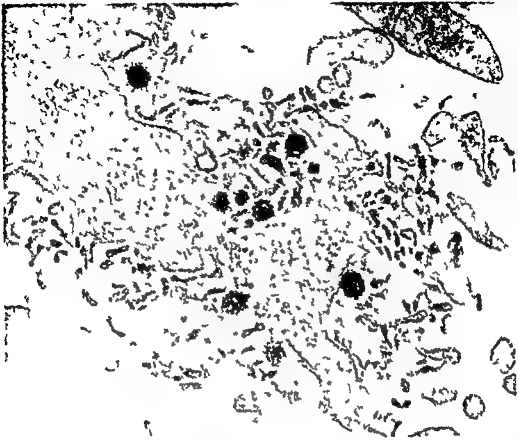


Fig 3 Portion of cell with extracellular HLV particles × 37,500

before culture and after establishment of the cell line showed no change over a 54 week study period The cells were positive for HL A 9 11 and 12 and negative for HL A 1 2 3 5 7 and 8

Cell Enzyme Studies

During culture the mean cell nitrogen doubled by 31 weeks and plateaued to three times preculture values at 79 weeks (table II) Cell aldolase activity initially in the low normal range increased by about 1 5 times in culture The cell pyruvate kinase activity low in the pre-culture peripheral blood lymphocytes showed an eightfold increase after 1 year in culture The hexokinase and glucose 6-phosphate dehydrogenase activities were low in the peripheral blood lymphocytes and in culture The phosphofructose kinase activity remained relatively stable and within the

Table II Leukocyte enzyme activity of peripheral blood lymphocytes in chronic lymphocytic leukemia in continuous culture

Weeks in culture	Mean cell nitrogen, μg N/cell $\times 10^{-6}$	Enzyme[1] activities, $m\mu M$/mg N/min at 30 C				
		HK	G-6-PD	PFK	Aldo	PK
Normal range	5 8–11.3	130–400	300–970	1 150–3 060	560–695	2,250–6,250
20	ND[2]	72	150	2,340	750	6,100
23	ND[2]	72	185	2,910	835	6,900
31	15 0	61	122	2,210	610	8 350
39	18 0	69	234	3,200	860	9 400
49	17 1	91	176	ND	790	8 020
54	21 8	72	204	2,290	730	8 950
64	17 0	80	212	2 950	620	9 300
79	23.2	74	233	3,200	850	[illegible]000
93	27 8	78	173	3,340	700	8,200
M G[3]	8 0	104	265	ND[2]	535	1,160
M G[4]	10 6	140	450	1,250	600	3,300

[1] HK = Hexokinase, G-6-PD = glucose-6-phosphate dehydrogenase PFK = phospho-fructose kinase Aldo = aldolase, PK = pyruvate kinase

[2] ND = Not done, these enzyme activities are calculated from observed activities of 10^6 cells and an assumed mean cell nitrogen content of 12 $\mu g \times 10^{-6}$

[3] Peripheral blood leucocytes 200 000/mm³, no previous treatment, in clinical relapse

[4] Peripheral blood leucocytes 30 000/mm³ On maintenance chlorambucil and in clinical remission

normal range during the time of culture The patient's peripheral blood lymphocytes tested after chemotherapy and at a time of clinical and hematological stability, showed normal levels of all enzyme activities tested

Discussion

Establishment of a long term peripheral blood lymphocyte culture from a patient with chronic lymphocytic leukemia (CLL) is unusual Previous and subsequent attempts to initiate long term cultures using the same techniques with lymphocytes obtained from ten other untreated patients with CLL, as well as from this patient, have failed A rising lymphocyte count and apparent increase in clinical activity of the disease alone are not enough to provoke establishment of an *in vitro* long-term

cell line The serum immunoglobulin patterns of the patients fail to indicate any characteristic that correlated with success or failure of long term cultures Establishment of this culture may have been related to the clinical status of the patient The lymphocytes were obtained at a time when the patient clinically had a vesicular herpetic eruption of the face and neck and an absolute lymphocyte count of 200 000 mm³ Although there was no rise in the herpes antibody titer, electron microscopic studies of the established cell line showed occasional HLV particles These particles in our experience [11] are usually found in established lymphoid cell lines

The ³H TdR flash labelling studies indicate that the generation time of these cells is greater than 24 h and less than 48 h These data are consistent with the observed range of doubling time of 30–50 h These observations are similar to those reported for other established lymphoid cell lines [23] Although acid hydrolase activity was not studied in the cultured cells euchrysine supravital staining studies demonstrated large numbers of cytoplasmic organelles (presumably lysosomes) within the cells in culture – especially in the larger cells Acid hydrolase activity has been reported to be decreased in CLL lymphocytes [28 31] and in mitogen stimulated CLL lymphocytes and this decrease in enzyme is associated with reduced numbers of lysosomes

The established cell line showed light microscopic as well as electron microscopic characteristics of lymphoid cells There was no characteristic change in the chromosomal mode which remained at 46 Aneuploidy and fuzziness in chromosome staining attested to a fragility and lability of the cellular DNA brought on by long term culture HL-A typing of the established cell line showed that these antigens were unaltered from the preculture cells

The observations on the enzyme activities as shown in table II are indicative *of the cell culture preference for glycolysis by the Embden Meyer*hoff pathway The mean nitrogen content of the cells in culture increased rapidly to three times that of the peripheral blood lymphocytes Hexokinase and G 6 PD enzyme activities on the other hand were well below those determined in the peripheral blood lymphocytes The PFK and aldolase activities remained within the high normal range but the pyruvate kinase enzyme activity was consistently high and almost seven or more times that obtained for the peripheral blood lymphocyte before culture and at least twice that seen in the peripheral blood lymphocytes of our patient after treatment and in clinical remission

These changes in enzyme activities and the increase in cell glycogen suggest an inhibition in *in vitro* cell glycolysis where glucose is the substrate as compared to the *in vivo* cell and an increase in reactions associated with glyconeogenesis via transamination. The very significant increase in PK and total cellular nitrogen would suggest a marked emphasis on the importance of products associated with reactions involving the Krebs cycle. This type of shift in the cellular metabolic pattern by lymphocyte-derived cells in culture as compared to the peripheral blood lymphocytes indicates an induced change necessary for successful cell survival in the *in vitro* state.

Long term lymphocyte cultures have been established previously from peripheral blood of patients with diseases in which there is an increased number of circulating lymphocytes in active DNA synthesis, i.e. hepatitis, infectious mononucleosis, herpes, or following phytomitogen stimulation [3, 8, 9, 14]. The response of lymphocytes from patients with chronic lymphocytic leukemia to mitogen stimulation is diminished in magnitude and delayed in time [26–29]. These observations fit into a concept of chronic lymphocytic leukemia as an accumulation of immunoincompetent [7] long lived cells with low proliferative properties.

Phytomitogen studies of peripheral blood lymphocytes made at the time of initial *in vitro* culture showed the peripheral blood lymphocyte response to phytohemagglutinin (PHA) was slightly delayed, but radiothymidine incorporation was equal to that of a paired control subject. Pokeweed mitogen (PWM) stimulation was not only delayed, but a very poor response peak was recorded. Minimal radiothymidine incorporation was found in simultaneously studied unstimulated lymphocytes. 5% of the peripheral blood lymphocytes from the patient formed rosettes with sheep red blood cells (SRBC R), a marker for thymus-dependent lymphocytes which are markedly reduced in most CLL patients [29, 30]. The established cell line showed only 1% of cells bearing a receptor for the third component of complement, a marker which is present on most circulating B lymphocytes [32]. Since recent studies [30–33] indicate the presence of cells bearing both B and T cell markers, as well as cells lacking these markers, our data do not permit classification of the cell of origin.

The reason for the successful establishment of this culture with failure of previous and subsequent attempts from patient M. G. and other patients is unknown. The relative ease with which this patient responded to therapy by complete clinical as well as bone marrow remission (at the time his peripheral blood contained cells capable of being established

long term culture) indicated that no prognostic value can be assigned to this event and implies that occasional blast cells in the peripheral blood of patients with chronic lymphocytic leukemia need not mean serious clinical consequences

Acknowledgement

We are grateful to Dr M Fotino of the New York Blood Center for performing the HL A typings and to Ms Ida Erlinger for the technical assistance in the leucocyte enzyme studies

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Acta Haemat. 52: 95–106 (1974)

Functional Characteristics of Chronic Monocytic 'Leukemia'[1]

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Abstract. A case suffering from a so-called pure monocytic leukemia displayed excessive monocytic hyperproliferation, moderate histiocytic infiltrations in lymph nodes and liver, hyperproliferation of plasma cells and monoclonal hypergammaglobulinemia of the IgG type. Monocytic cells proved to be normal with respect to morphology including electron microscopy, cytochemistry, capacity of phagocytosis, motility and differentiation potential. An increased fraction of immature monocytic cells gained access to the blood. The immature cells being able to egress from the marrow proved incapable to leave the microcirculation. They continued to circulate until completion of differentiation to mature monocytes which finally developed a normal emigration potential.

Key Words
Cell kinetics
Monocytes
Monocytic leukemia
Monocytopoiesis
Preleukemia

This paper intends to further characterize the so-called chronic pure monocytic leukemia of Reschad-Schilling type [15]. The study bases upon the present knowledge of the life cycle of monocytic cells in man [10] and a variety of analytical methods including electron microscopy, cytochemistry and cell kinetic investigations.

Materials and Methods

Case history. The patient (M. J., J. No. 43 22171 8) was a 77-year-old cartwright whose medical history did not reveal any remarkable prior disease. He was admitted to the hospital due to furunculosis. Physical examination disclosed several moderately enlarged axillary and inguinal lymph nodes. The liver edge could

[1] Supported by Muller-Fahnenberg Donation and Swiss Cancer League, grant No. FOR. 034.AK.73 (1).

Table I Quantitative analysis of serum proteins, mg/100 ml

	Patient	Normal, means ± SD
Total protein	9,000	7,290 ± 330 [7]
Albumin	3,240	3,990 ± 90 [7]
α_1-globulin	270	380 ± 90 [7]
α_2-globulin	360	650 ± 90 [7]
β-globulin	540	850 ± 110 [7]
γ-globulin	4,590	1,420 ± 150 [7]
IgA	750	210 ± 82 [1]
IgM	270	125 ± 53 [1]
IgG	5,225	1,250 ± 280 [1]

Table II Myelogram, %

	Patient	Mean of 10 normal individuals
Erythroblasts	14.3	28.0
Granulopoietic cells	33.7	50.6
Promonocytes		
All forms	42.1	2.9[1]
Round or oval nuclei	21.8	1.0[1]
Slightly folded nuclei	13.8	1.5[1]
Distinctly folded nuclei	6.5	0.4[1]
Lymphatic cells	3.8	8.0
Plasma cells	5.7	1.4

[1] Determined by demonstration of NaF-resistant and NaF-sensitive naphtol AS D-acetate esterases

be palpated 3 cm below the right costal margin. Spleen size, measured by radiography, was normal. Histology of an excised enlarged lymph node demonstrated histiocytic infiltration. Histology of a liver biopsy showed periportal and intrasinusoidal histiocytic infiltrations.

The patient was observed over a period of 7 months. He thereafter suddenly died at home from cardiac insufficiency. Within this time span he suffered from three consecutive infections (furunculosis and twice bronchopneumonia). The hematological status remained relatively constant demonstrating moderate anemia (10.1–12.2 g Hb/100 ml) due to hemolysis (bilirubin 1.8 mg/100 ml) and inadequate erythropoietic compensation (reticulocyte count about 150,000/μl). Thrombocyte counts were intermittently depressed to 20,000/μl. Neutrophils and lymphocytes

were normal, both with respect to blood counts and cytochemistry (table IV). Cytogenetic studies in bone marrow cells revealed no chromosomal abnormality. Lysozyme activity in serum, determined by an agar plate method [14, 17], was increased to 116 µg/ml (normal 23±6 µg/ml [17]) and in blood leukocytes to 12.6 µg/10^6 leukocytes (normal 7–10 µg/10^6 leukocytes [17]). Analysis of serum proteins (table I) revealed a marked monoclonal hypergammaglobulinemia of IgG type. Serum albumin level was slightly reduced, IgG concentration was 4 times higher than normal. No proteins could be detected in the urine. There was a rise in morphologically normal plasma cells in bone marrow smears (table II). Plasma cells also occurred in the circulating blood (about 30/µl).

Morphological and cytochemical investigations. The myelogram was established by differentiating 1000 May-Grünwald-Giemsa stained nucleated cells. Differential blood counts were performed by differentiating 400 leukocytes. Morphology and cytochemistry of blood monocytes were investigated in concentrated leukocyte smears [12] which were submitted to the following procedures: May-Grünwald-Giemsa stain, demonstration of naphthol AS-D-chloroacetate esterase [4], peroxydase [3], Sudan black B [6], acid phosphatase [2], NaF-resistant and NaF-sensitive naphthol AS-D-acetate esterase [16] and PAS [8]. 500 monocytes were scanned for each cytochemical analysis. The cells were classified into three groups according to nuclear morphology: monocytes with large round or oval nuclei, monocytes with reniform or slightly folded nuclei, and monocytes with distinctly folded nuclei. Intensity of the different cytochemical reactions are reported as activity indices, established by ranking the monocytes into four semiquantitative categories: 0 = negative, 1 = slightly positive, 2 = moderately positive and 3 = strongly positive. The reaction score was multiplied with the corresponding cell number and the reaction index expressed by the sum of the products of 100 cells.

Cell kinetic studies. Blood monocyte kinetics were investigated by transfusion of 500 ml autologous blood which had been previously incubated *in vitro* with 750 µCi ^{3}H-diisopropyl fluorophosphate (^{3}H-DFP). At suitable intervals after completion of autotransfusion venous blood samples were taken for preparation of concentrated leukocyte smears. After autoradiography and Giemsa staining, labeling indices of blood monocytes were determined microscopically by counting 1,500 monocytes. Details of the method have been described elsewhere [12].

In an attempt to obtain information about the kinetics of monocytopoiesis the chronology of labeled monocytes circulating was examined following ^{3}H-thymidine (^{3}H-TDR) pulse labeling. 0.1 mCi ^{3}H-TDR/kg body weight were injected intravenously. Subsequently, at 2-hourly intervals, concentrated leukocyte smears were prepared from 4 ml of venous blood [12]. The stripping film method (Kodak AR 10) was used for autoradiography, the exposure time being [illegible] days. For each sample silver grains of 1,500 monocytes with round or oval nuclei and 1,500 monocytes with distinctly folded nuclei were counted.

Mikrokinematographic studies. Drops of leukocyte-rich plasma (prepared from fresh blood with heparin after 30 min sedimentation) were placed between a slide and a coverslip and the preparation sealed with paraffin. Monocyte mobility was observed using a phase contrast microscope (40-fold magnification) in a temperature controlled cage at 37 °C, electronic flash and automatic camera, taking 10 pictures/min.

Results

Monocytopoiesis and Blood Monocytes

Bone marrow smears were hypercellular due to tightly packed promonocytes. Their relative number was about 15 times higher than normal (table II). Blood monocyte counts ranged between 5,000 and 20,000/μl (fig 2). The monocytic cells observed in bone marrow and peripheral blood demonstrated similar morphological characteristics. However, the frequency distribution of the different cell types was inverse (tables II, III). Cells with round or oval nuclei for instance, representing the largest fraction in the patients' bone marrow were only rarely found in the blood. On the other hand, cells with distinctly folded nuclei, representing the smallest fraction in the marrow, represented the main fraction in the blood.

Morphological evaluation using light and electron microscopy was not able to reveal deviations from normal monocytic cells (fig 1). The different cell types demonstrated avid phagocytosis of carbon particles. Microkinematographic observations in living preparation showed active cytoplasmatic and nuclear movements being very sluggish in cells with round or oval nuclei, increasing with the development of folded nuclei. Nuclear movements caused reversible transitions between round-to-oval and slightly folded nuclei on one hand and transitions between slightly folded and distinctly folded nuclei on the other. In culture, blood mono-

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Fig 1. Monocytic cells were divided into three types according to the shape of the nuclei: cells with round to oval nuclei, cells with slightly folded nuclei and cells with distinctly folded nuclei. × 9,500. *a* Electron microphotograph demonstrating homogenously dispersed chromatin in a monocyte with oval nucleus (N). The cytoplasma of this immature cell contains large promyelocyte-like granules (indicated by arrow) and numerous small electron-dense granules. The nucleus is surrounded by rough endoplasmic reticulum (FR). The cell membrane displays few finger-like projections. *b* A monocyte exhibiting an indented nucleus (slightly folded nucleus) is characterized by accumulation of chromatin along the nuclear membrane, predominance of small electron-dense granules and only few large granules (arrows), a well developed Golgy complex (G), fibrils (F) in a juxtanuclear position, some short strands of rough endoplasmic reticulum (FR) and numerous mitochondria (M). *c* The electron micrograph of a cell which displays a distinctly folded nucleus and which corresponds to a normal mature monocyte: dense chromatin, small electron-dense granules, a few short strands of rough endoplasmic reticulum, vacuoles, and abundant prominent finger-like projections on the cell surface.


ER
N
a
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F
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G
b
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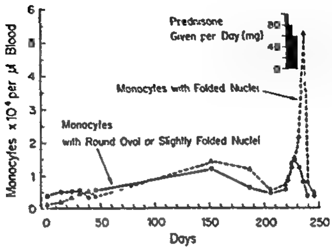


Fig 2. Monocyte blood counts during an observation period of 7 months. Effect of prednisone administered orally.

cytes differentiated into macrophages. The *in vivo* counterpart reflecting normal differentiation potential of monocytic cells was the cytology of pleural exudate, exhibiting a uniform picture of mature macrophages.

The cytochemical reactions in the patients' blood monocytes corresponded to those of blood monocytes in healthy individuals (table IV). Monocytes with round or oval nuclei exhibited the lowest reaction intensity of NaF-sensitive naphthol-AS-D-acetate esterase combined with the highest activity of naphthol-AS-D-chloroacetate esterase and peroxydase. Blood monocytes with distinctly folded nuclei had an inverse pattern whereas the reactions of monocytes with slightly folded nuclei fitted in between of these two groups.

Cell Kinetics

Intravascular kinetics of monocytic cells were studied by autotransfusion of blood cells previously labeled *in vitro* with ^{3}H-DFP. Figure 3 illustrates the disappearance of labeled monocytes from the circulating blood. Biphasic curves resulted from both, monocytes with round or oval nuclei and monocytes with distinctly folded nuclei. The initial rapidly declining component gradually converted into a slowly declining exponential curve. This second component was used for graphical determination of the cells' half-disappearance time ($T^1/_2$). $T^1/_2$ reached a value

Table III Blood monocytes

	Blood monocyte count/µl	
	patient[1]	mean of 10 healthy individuals
Total	8 440	370
Monocytes with round-oval nuclei	1 850	30
Monocytes with slightly folded nuclei	2,100	120
Monocytes with distinctly folded nuclei	4 450	220

[1] Mean values of 13 determinations within an observation period of 7 months

Table IV Activity indices of cytochemical reactions of blood leukocytes

	Monocytes			Neutro-phils	Lymphocytes
	round-oval	slightly folded	distinctly folded		
NaF-sensitive naphtol-AS-D-acetate esterase	50 (73 ± 36)	72 (134 ± 26)	151 (141 ± 33)	0	0
Naphtol-AS-D-chloroacetate esterase	171 (128 ± 58)	80 (53 ± 21)	11 (26 ± 11)	153	0
Peroxydase	63 (114 ± 31)	4 (68 ± 31)	0 (59 ± 21)	233	0
Sudan black B	105	43	12	259	0
PAS	40	7	4	296	36
Acid phosphatase	3	75	[illegible]	0	39
Neutrophil alkaline phosphatase	0	0	0	132	0

Data in parentheses represent the mean values and standard deviations of 10 healthy individuals

of about 80 h for monocytes with round or oval nuclei and a value of 14 h for monocytes with distinctly folded nuclei.

Figure 4 represents the behavior of labeled monocytic blood cells after intravenous injection of a single dose of ^{3}H-TDR (nuclei overlayed by more than two grains in the autoradiographs were counted as labeled). One hour after injection of ^{3}H-TDR blood monocyte labeling in-

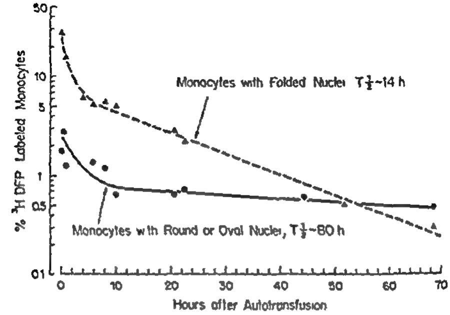


Fig 3 Intravascular behavior of ³H DFP labeled autologous monocytes following transfusion

dex was about 8%. The fraction of labeled cells with round or oval nuclei exhibited a rapid initial increase and subsequent oscillation of the values around a rather constant level. In contrast to this the labeling indices of monocytes with distinctly folded nuclei steadily increased after an initial delay. Mean grain counts of labeled monocytes with round or oval nuclei initially increased to a peak and thereafter gradually decreased. The grain counts of labeled monocytes with distinctly folded nuclei, being very low during the first 10 h, gradually rose and finally paralleled the values of cells with round or oval nuclei.

Systemic administration of prednisone to the patient caused an increase of blood monocyte counts (fig 2). The rise was markedly higher in monocytes with distinctly folded nuclei than in the other cell types.

Discussion

The numbers of monocytic cells in bone marrow and peripheral blood exceeded the normal values by factors of 15–20. The cells did not

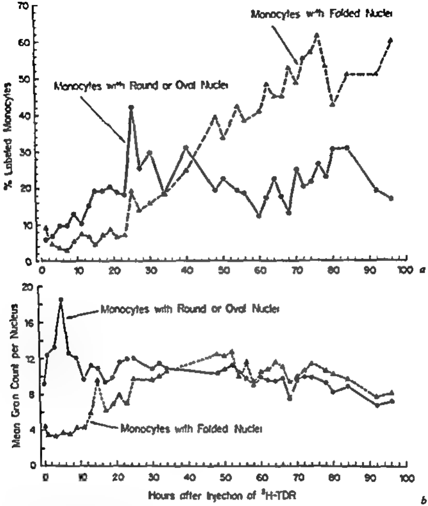
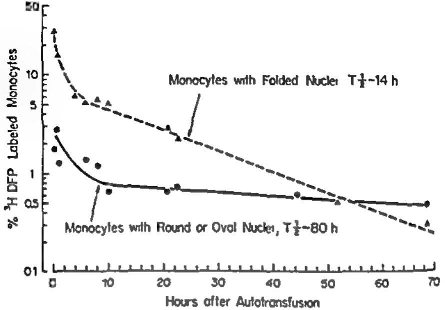


Fig. 4. Behavior of labeling indices and mean grain counts of blood monocytes following ^{3}H TDR pulse labeling

deviate from normal monocytic cells with respect to morphology, cytochemistry, motility, phagocytic activity and differentiation potential. As observed in monocytic cells from healthy individuals [5, 11] the differentiation process of the patients' monocytes was also paralleled by an



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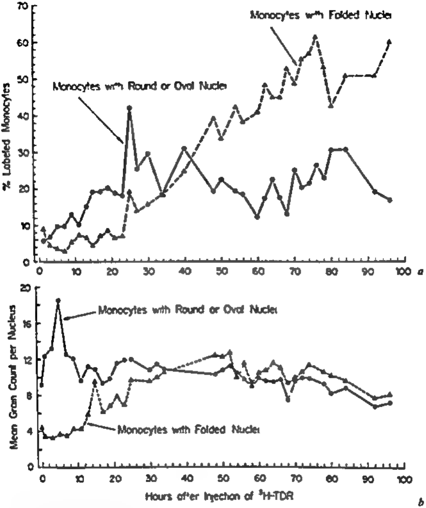

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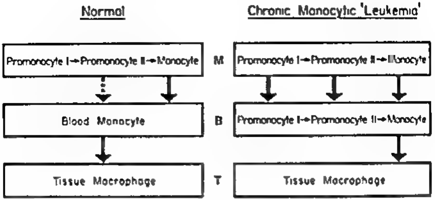

Fig 5 Flux of monocytic cells from the bone marrow (M) into the blood (B) and from the blood into the tissue (T) In the patient large amounts of immature monocytic cells gained access to the blood These immature cells being able to egress from the marrow proved incapable to leave the blood vessels They continued to circulate and to complete differentiation to mature monocytes which finally developed a normal emigration potential

increase in cytoplasmatic and nuclear motility, development of folded nuclei and a rise in activity of NaF-sensitive nonspecific esterases Therefore, cells with round or oval nuclei were regarded as immature promonocytes, whereas cells with distinctly folded nuclei as mature monocytes

In circulating blood of healthy individuals monocytes with round or oval nuclei, monocytes with slightly folded and those with distinctly folded nuclei had a mean frequency distribution of 8 32 60% [11] In the patient the frequency of these three cell types was 22 25 53% This indicates that a higher fraction of immature monocytic cells was released into the blood A similar 'shift to the left' has also been observed in cases with benign monocytosis [11]

Enhanced marrow egress of immature monocytic cells might account for the abnormal pattern of labeled blood monocytes observed after ^{3}H-TDR pulse-labeling (fig 4) In normal individuals ^{3}H-TDR pulse labeling produces several discrete waves of labeled monocytes entering the blood These waves reflect the temporal sequence and duration of the DNA synthesis phases within the proliferating precursor compartment They arise from an orderly progression of labeled cells through the proliferating pool and predominance of mature cells being released into the blood (fig 5) The difference in curve characteristics between ^{3}H-TDR

labeled immature and mature monocytic cells may indicate an intravascular cell maturation. This process would give rise to migration of the marker from monocytes with round or oval nuclei to monocytes with distinctly folded nuclei and might have caused both, the observed intravascular accumulation of labeled mature monocytes and as well as the gradual adjustment of the mean grain counts of mature and immature monocytes.

An extremely prolonged intravascular half-disappearance time of about 80 h was observed in monocytic cells with round or oval nuclei. This indicated that the majority of these immature cells is incapable to leave the microcirculation. Therefore they probably continue to circulate until completion of maturation to monocytes with distinctly folded nuclei (fig. 5). The half-disappearance time of this latter cell type was 14 h. This value lays within the range of monocyte half disappearance times observed in patients with benign monocytosis and slightly above normal (4.5–10.0 h [12]). Considering that the loss of labeled mature monocytes was concomitted by a simultaneous production of labeled mature cells, the half-disappearance time of 14 h indicates a normal potential of the mature cells to penetrate the vessel walls. Administration of prednisone might have reduced the capability of monocytes to leave the vessels with consequent intravascular cell accumulation (fig. 2). It is interesting to note that the count of immature monocytes peaked prior to that of mature ones. This phenomenon might also have resulted from intravascular cell maturation and from a predominance of mature monocytes immigrating into tissues.

In conclusion, our investigations in this patient were not able to detect qualitative but marked quantitative alterations of the monocytic system. Therefore, it is equivocal whether this disorder represents a true neoplasia. The findings would be consistent with the concept of an extreme degree of benign monopoietic hyperproliferation. In addition, the disorder comprised a marked hyperproduction of plasma cells and immunoglobulins, thus confirming the statement of OSSERMAN [13] of an interrelationship between monocytic and plasmacytic dyscracias. Both cell systems cooperate in immune defense which was probably impaired in the patient as indicated by the high susceptibility to infection. Bearing in mind that monocytic disorders of this kind were observed to transform into acute leukemia [18] one may ask whether the entity represents a preleukemic condition or a reaction against a leukemogenic factor affecting the organism.

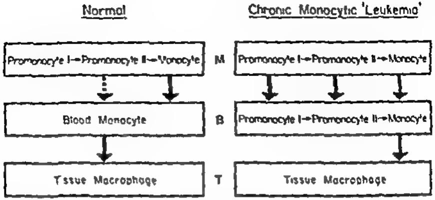

Fig. 5. Flux of monocytic cells from the bone marrow (M) into the blood (B) and from the blood into the tissue (T). In the patient large amounts of immature monocytic cells gained access to the blood. These immature cells being able to egress from the marrow proved incapable to leave the blood vessels. They continued to circulate and to complete differentiation to mature monocytes which finally developed a normal emigration potential.

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Acta Haemat 52 107–111 (1974)

IgG Myeloma, Sia Test, and Serum Hyperviscosity

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Abstract A case of λ type IgG myeloma with serum hyperviscosity a strong positive Sia test and few clinical manifestations is described. As the M-component concentration is not excessively high after study by thin layer chromatography these phenomena are attributed to the molecular shape and probably (although in a lesser degree) to M-component aggregation

Key Words
Immunoglobulin
Myeloma protein
Serum viscosity
Sia test

It is well known that a hyperviscosity syndrome can complicate the course of IgG myeloma, 61 cases have been reported between 1965 and 1972 [5] and two new cases have been added afterwards [7, 9]

In this report we describe a case of λ type IgG myeloma with serum hyperviscosity and a strongly positive Sia test in a patient with a good general condition and few clinical manifestations of the hyperviscosity syndrome

Case Report

A 67 year-old man was admitted to the Surgery Department for revision of a peptic ulcer For the previous 3 months suffered from blurred vision In the examination the existence of an M-component in the serum electrophoresis was discovered so he was transfered to the Department of Internal Medicine The patient's general condition was good temperature and blood pressure were normal The liver spleen and lymphatic nodes were not increased in size The eye fundus showed engorged sausage like veins without exudates or hemorrhages The neurological examination was normal The chest and bone X rays were normal

Laboratory Data Haematocrit 49%, haemoglobin 15 g%, leukocytes 6 600 $/\mu l$ (45% segmented, 5% bandform neutrophils 1% eosinophils, 43% lymphocytes and 6% monocytes) Platelet count 100 000$/\mu l$ Erythrocyte sedimentation rate 130 mm in 1 h Blood glucose 66 mg total bilirubin 0 40 mg creatinin 1.20 mg serum uric acid 4 8 mg total cholesterol 170 mg, phosphorus 4 1 mg and

Fig 1 Serum (above) and Sia precipitate (below) electrophoresis an M-component in the slow γ region is observed

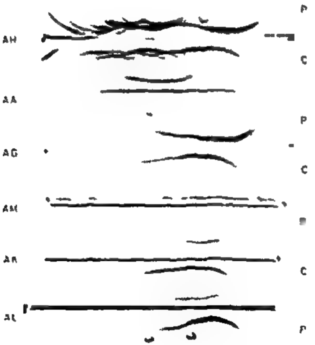

Fig 2 Serum immunoelectrophoresis The M-component is identified as a λ type IgG P = Patient C = control AH = total human antiserum AA = anti IgA antiserum AG = anti IgG antiserum AM = anti IgM antiserum AK = anti K antiserum AL = anti λ antiserum

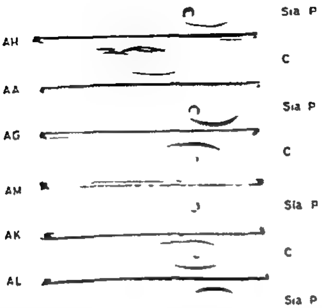

Fig 3 Immunoelectrophoresis of the Sia precipitate which is identified as a κ type IgG Sia P = Sia precipitate C = control AH = total human antiserum, AA = anti IgA antiserum AG = anti IgG antiserum AM = anti IgM antiserum AK = anti K antiserum AL = anti λ antiserum

8.3 mg/100 ml Electrolytes acid base state and serum enzyme activity were normal Rheumatoid factor was not present Urine sediment was normal The bone marrow aspirate revealed 42% immature plasma cells

Protein studies Total serum proteins 9.9 g/100 ml Electrophoretically a homogeneous peak was detectable in the slow γ region (fig 1) Serum albumin 3.6 g/100 ml M-component 3.9 g/100 ml Immunoelectrophoretic analysis of the serum revealed that the M-component was constituted by κ type IgG (fig 2) the IgM and IgA being decreased The Sia test, carried out in deionized water at pH 5.5, was strongly positive (4+) in pH 7.2 it was also positive but to a lesser degree (2+) The Sia precipitate was isolated after rinsing it three times with distilled water and redissolving it in a 0.15 M solution of NaCl alcalinized with 0.1 N NaOH to pH 8 Electrophoresis and immunoelectrophoresis (fig 1 3) showed that it was practically exclusively constituted by the same κ type IgG component found in the serum

The serum and Sia precipitate were studied on thin layer gel chromatography on Sephadex G 200 (superfine) with a 0.1 M Tris HCl buffer which contained 1 M NaCl in pH 8 The M-component behaved as the substances of a molecular weight of 7S

Fig 4 Thin layer chromatography on G 200 Sephadex The M component migrates with the 7S molecular weight substances with a small trail in the serum between the 7S fraction and the one corresponding to the 19S fraction C = control P = Patient serum Sia P = Sia precipitate

even though one could observe a trail in the serum which reached the faster migration fraction corresponding to the substances of a molecular weight of 19S (fig 4)

There were no cryoglobulins Bence Jones protein in urine was negative and the relative serum viscosity measured with Hess viscosimeter, was 3.5

Discussion

The discovery of an M component in the serum of asymptomatic individuals in early phases of myelomatosis is not an exception [8] In our case the presence of an IgG M-component with decreased IgA and IgM together with proliferation of a large number of immature plasma cells in the bone marrow, allows a diagnosis to be made before the general condition is affected

Serum hyperviscosity in IgG myeloma has been attributed to aggregation of 7S components [1] to the high concentration of serum M-component, and to the molecular shape of immunoglobulins [4] In our case M component concentration is not excessively high, and the existence of

circulating aggregates as the only or principal cause of hyperviscosity can be ruled out, because the M-component of both complete serum and Sia precipitate behaved as a 7S component during thin layer chromatography on G 200 Sephadex. Although the presence on chromatography of a small trail between the 7S components and those which emigrate first (19S components) could indicate a small proportion of IgG aggregates. This fact could be related to the strong positivity of the Sia test at pH 5.5 and to a lesser degree at pH 7.2. This is in accordance with the discoveries of MARTIN [3] and FRANGLEN [2] who relate precipitation intensity of the different globulin fractions to the pH of the medium in which it is carried out.

Although in our case the serum hyperviscosity was not excessively high and only a few clinical manifestations of hyperviscosity were present, the positive Sia test should probably be related to the molecular shape of the M-component and to the small proportion of IgG aggregates. These coinciding phenomena, on the other hand do not maintain a significative correlation in many of the cases reported in the literature [6].

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Acta Haemat 52 112–119 (1974)

Spontaneous Haemophilia in a Genotypically Normal Female

A Family Study

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Abstract The diagnosis of spontaneous haemophilia A with severe antihaemophilic globulin deficiency (less than 1%) was established in an 11 year old girl with a normal female chromosomal pattern Coagulation studies proved that the proposita is the offspring of a normal father with negative history for bleeding disorders and a normal mother who had an antihaemophilic globulin concentration of 200% Family history revealed no bleeding disorder in 3 successive generations of the maternal side This together with AHF activity exceeding slightly AHF like antigen concentration provides good evidence against the carrier state of the mother The possible mechanisms through which spontaneous haemophilia may occur in a genotypically normal female are discussed

Key Words
Bleeding disorders
Factor VIII deficiency
Haemophilia in females

Although haemophilia in females has been the subject of a rapidly increasing number of reports in recent years [1–3, 5, 8–10, 13–15, 17, 19, 20 22, 23 25 27] the number of reported cases of spontaneous haemophilia in karyotypically normal females is very small Out of the 24 cases of true haemophilia reported in human females, only 4 cases of true sporadic haemophilia have been described in apparently normal females not verified by chromosomal studies [1, 10] The remaining 20 cases belong to two main groups the first group consists of 7 cases who are truly homozygous for the classic sex linked recessive haemophilia gene and are the product of bleeder-conductor marriage and the second group includes 13 cases who are assumed to be heterozygous for the abnormal gene which is inherited from the mother The fact that the latter group exhibits all clinical manifestations of haemophilia is explained either on the basis of chance mutation producing a haemophilic gene on

the X-chromosome inherited from the father, or on the basis of LYON's hypothesis [11, 12] which speculates that in such cases inactivation of the majority of the paternal normal X-chromosomes has occurred in embryonic life

It is the purpose of this paper to report on an 11-year-old genotypically normal girl with severe haemophilia A, whose pattern of inheritance is unusual in the fact that she is the offspring of a normal father and non-carrier mother whose AHG level is 200% and whose AHF activity slightly exceeds AHF-like antigen concentration As double chance mutation at conception is not compatible with the low mutation rate of sex linked recessive traits in man, other hypotheses for the explanation of the occurrence of spontaneous haemophilia in genotypically normal females are put forth

Case Report

N D., an 11 year-old girl presented in the out patient department in 1964 at the age of 3 years with haemarthrosis of the left ankle joint and a tendency to easy bruising from the age of 6 months. She was hospitalized on 3 occasions because of severe attacks of bleeding from the tongue which lowered her haemoglobin level to 3 7 g/100 ml She had repeated haemarthroses which had always been associated with mild trauma and had usually involved the left ankle and left elbow joints. At the age of 8 years she was admitted to hospital for further investigation of her bleeding disorder

Physical examination revealed a well built phenotypically normal girl with numerous ecchymoses over the limbs. She had a mild flexion deformity of the left elbow and partial ankylosis of the left ankle, but other joints were normal She had normal female genitalia and no testes or lumps could be felt in the groins. All other physical findings were normal Intravenous *pyelogram* showed normal kidneys, ureters and urinary bladder

Family history The parents deny any consanguinity There is no history of bleeding disorder in the family of the father who has 2 brothers and 2 sisters, and their coagulation studies were all normal The mother is the youngest of 3 siblings, the oldest is a male whose 3 male offsprings underwent circumcision uneventfully, and the middle is a female whose only daughter showed normal screening coagulation tests.

The maternal grandfather had an uncomplicated prostatectomy and the maternal grandmother gave no history of bleeding trouble after deliveries or during menstrual periods and she never complained of menorrhagia. The patient is one of 3 siblings, a brother who underwent *uneventful* circumcision and tonsillectomy operations and a sister who exhibited no *abnormal bleeding* tendency This detailed study of the maternal side of the pedigree *provides good* evidence against the possibility that the mother may be a carrier through inheritance

Table I Results of coagulation studies on the proposita and her family members

	Coagulation time min	Bleeding time min	Platelet count per mm³	PTT sec	TGT abnormality in	F VIII %	F IX %	Prothrombin time sec
Normal values	5–11	7	140 000 459 000	35		50–200	100	13
Proposita	14	5	235 000	82	plasma	1	170	14
Father	5	4	185 000	36	normal	–	–	13
Mother	6	3	220 000	33	normal	200	85	14
Sister	5	5	190 000	34	normal	[illegible]	–	13
Brother	4	4	165 000	35	normal	92	–	14
Maternal uncle	6	5	230 000	34	normal	100	–	14
Maternal aunt	5	4	175 000	36	normal	[illegible]	–	14
Maternal cousin	6	5	210 000	35	normal	120	–	14
1	5	4	220 000	33	normal	80	–	13
2	6	6	170 000	34	normal	95	–	14
3	5	5	195 000	36	normal	105	–	13

Coagulation studies All coagulation tests were carried out as described by DACIE and LEWIS [5] Coagulation times were determined by the Lee and White method and bleeding times were measured by Ivy's technique Platelets were counted by the direct method using formol citracte as diluting fluid Partial thromboplastin times with kaolin activation were done according to the method of PROCTOR and RAPPAPORT and thromboplastin generation tests as outlined by BIGGS and DOUGLAS antihaemophilic globulin activity and Christmas factor activity were assessed by the one stage method. The results of the tests performed on the proposita and the family are outlined in table I

The immunoassay study of the relationship between functional anti haemophilic factor (AHF) and AHF like antigen carried out on the plasma of the proposita and her mother provided a more sensitive laboratory means of excluding von Willebrand's disease in the former and confirming the non-carrier state in the latter The immunoassay of functional AHF activity and AHF like antigen concentration was carried out as described by ZIMMERMAN *et al* [26] Since the immunological estimations of AHF like antigen in unconcentrated plasma were neither sensitive nor reproducible all these studies were carried out on ethanol concentrate of plasma The amount of antigenic material present in ethanol concentrates of pooled plasma freshly drawn from 20 normal adult males was arbitrarily defined as 1 0 antigen/ml

Whereas the arithmetic mean of the antigen concentration in the ethanol concentrate of plasma of 20 normal persons is 0.9 antigen U/ml (SD ± 0.3) its concentration in ethanol concentrate of the patient's plasma is 0 95 U ml providing an unequivocal evidence against the diagnosis of von Willebrand's disease in the girl

Table II Erythrocyte and haptoglobin (H^+) typing

	A	B	C	D	E	c	e	N	M	S	P_1	K	Lu^a	Le^a	Le^b	Fy^a	H^+
Patient	−	+	+	+	−	+	+	+	+	−	+	−	−	−	−	−	2-1
Mother	−	+	−	−	−	+	+	+	+	−	+	−	−	+	−	−	2-2
Father	−	−	+	+	−	+	+	+	+	−	+	+	−	−	−	−	2-1

H^+ = Haptoglobin

Table III AHG level before and after cryoprecipitate infusion

Date	Amount of cryoprecipitate infused units	AHG levels, %	
		before infusion	after infusion
[illegible] Nov 1970 8 a.m.	4	1	36
26 Nov 1970			
8 a.m.	4	8	43
9 p.m.	4	20	48
27 Nov 1970			
9 a.m.	4	17	50
9 p.m.	4	18	44
[illegible] Nov 1970			
11 a.m.	4	20	54
9 p.m.	4	23	49
29 Nov 1970			
11 a.m.	4	[illegible]	50
9 p.m.	4	21	42

The immunoassay of the antigen in the ethanol concentrate of the mother's plasma on 3 successive days revealing a mean concentration of 0.85 U/ml compared with mean AHG activity of 1.1 U/ml provides a strong evidence against the carrier state of the mother.

Chromosomal studies conducted on the peripheral blood leucocyte culture indicated that the chromosomes were normal in number and no mosaicism was present. The relatively low number of Barr bodies in the buccal smear lent some support to the possibility of gonadal dysgenesis, but the complete absence of all clinical manifestations of the gonadal dysgenesis syndrome such as short stature, neck webbing and visceral anomalies should exclude this possibility.

Erythrocyte and haptoglobin typing for the proposita and her parents provide no evidence to dispute paternity (table III), as none of the red cell antigens detected in the patient's red cells as well as her haptoglobins could not be accounted for by inheritance from both parents

Other Investigations As sporadic haemophilia in a normal female is extremely rare One has to verify two main facts first one has to prove that antihaemophilic globulin deficiency is not a manifestation of von Willebrand's disease or one of its variants, secondly one has to demonstrate a normal female chromosomal pattern in the patient To exclude von Willebrand's disease as a basis for the haemostatic defect, Ivy's bleeding time was repeatedly done on many occasions and it always proved to be within normal limits in the proposita her parents, her brother and sister As bleeding time may be normal in some variants of von Willebrand's disease rapid intravenous infusion of 4 U of cryoprecipitate every 12 h over several days were carried out and the effect of the antihaemophilic globulin level was studied As shown in table III the infusions did not produce the exaggerated and protracted rise in AHG levels usually obtained in von Willebrand's disease, but resulted in a rise and fall in the AHG concentration to the levels expected from the usual half life of transfused factor VIII in the circulation of haemophiliacs Patient's platelet adhesiveness, measured according to the *in vivo* method of DIMITRIEM and BUNTING [7] proved to be normal

Discussion

The clinical data and the results of the laboratory investigations of this girl satisfy the requirements for the diagnosis of haemophilia A with antihaemophilic globulin (AHG) deficiency of the severe type As the patient is a female, the alternative diagnosis of von Willebrand's disease which affects both sexes and manifests AHG deficiency was excluded Laboratory tests performed for this purpose were Ivy's bleeding time which gave invariably normal results, and plasma AHG assays after cryoprecipitate administration which revealed a pattern of rise and decline of AHG level compatible with the rate of decay of AHG in haemophiliacs This contrasts with the *abnormal Ivy's bleeding time* in von Willebrand's disease together with the exaggerated and protracted rise in AHG level in response to AHG administration [4, 18]

The genetic mechanism responsible for the development of haemophilia A in the present patient is obscure as she is the offspring of a normal father and a normal mother who is most probably non-carrier on pedigree basis Haemophilia cannot be the result of a single mutation on one of the X-chromosomes since karyotype analysis excluded X-chromosomal abnormalities which may lead to full expression of a single haemophilic gene such as the XO karyotype of Turner's syndrome [21]

the XX/XO mosaicism [16] and the deletion of the portion of normal X-chromosome carrying the gene for AHG production [17] In view of the fact that double mutation is not compatible with the estimated mutation rate in human disease, it is reasonable to speculate that the girl's mother is a spontaneous carrier who has passed her defective X-chromosome to the daughter and that chance mutation at conception has involved the paternal X-chromosome The carrier state of the mother is suggested on purely speculative basis since there is no physiological or biochemical test for the clear-cut identification of heterozygosity in haemophilia.

This suggestion would be more acceptable in our case if an explanation is provided for the apparently paradoxical situation of a high AHG level of 200% in heterozygous female In the light of LYON's principle [11, 12] which postulates that in the female XX somatic cells, only one X-chromosome is functionally active and the other one is genetically inactive, a reasonable explanation can be provided by speculating that chance inactivation has involved the defective X-chromosomes in almost all the mother's cells, allowing full expression of the unopposed gene responsible for AHG production, thus accounting for her AHG level of 200% Although according to LYON's principle, the distribution of inactivation follows a statistically random pattern resulting in inactivation of the defective and normal X-chromosomes in virtually equal proportions, a minority of cases do have the defective or the normal X-chromosomes inactivated in almost all cells

One can, according to LYON's principle, offer an explanation for the development of haemophilia in this girl in a different way If the speculative explanation of the heterozygous state of the patient is accepted, it can be assumed that the present patient belongs to the small group of heterozygote females who have their normal X-chromosomes inactivated in almost all the somatic cells In this way the patient's cells will be identical to those of the hemizygous affected male who carries only one defective X-chromosome and exhibits all clinical and laboratory manifestations of haemophilia

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Acta Haemat. 52 120-125 (1974)

Disseminated Intravascular Coagulation during a Fatal *Mycoplasma pneumoniae* Infection

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Abstract. A case of disseminated intravascular coagulation (DIC) during a fatal *Mycoplasma pneumoniae* infection is reported. The results of several laboratory investigations suggested the presence of an autoimmune abnormality. Recurrence of DIC in the final period may have been due to circulating complement activating immune complexes.

Key Words
Coagulation disorders
Disseminated intravascular coagulation
Immune complexes
Mycoplasma pneumoniae

Respiratory infections caused by *Mycoplasma pneumoniae* usually have a benign course. An overwhelming illness with fatal outcome rarely occurs [4, 7–10]. Bacterial and viral infections are sometimes complicated by disseminated intravascular coagulation (DIC) [11]. Although hematological complications, e.g. hemolytic anemia, may occur in the course of *M. pneumoniae* infections [6], to our knowledge DIC has not been described. We should now like to report the occurrence of DIC in a case of *M. pneumoniae* infection.

Case Report

A 37-year-old man was admitted to the intensive care unit because of hypotension and purpura. Six months previously he complained of fatigue and a disturbed carbohydrate tolerance was found. A diet was prescribed and the patient continued his activities. The day before admission he complained of extreme weakness and myalgia. He had a temperature of 40 °C and was treated with aspirin and penicillin. In the morning of December 7, 1972 he still had fever and purpuric spots were seen on the chest and neck. In the afternoon hypotension occurred, the patient being transferred to the hospital.

[1] We thank Prof. E. Veys for the determinations of IgG, IgA, IgM and C_3 levels and Prof. F. Barbier for his advice and the criticism in the preparation of this paper.

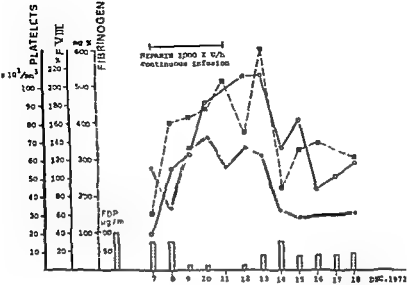

Fig 1 Blood coagulation parameters platelets (●) factor VIII activity (○,) fibrinogen (■) and serum FDP concentrations (columns, μg/ml)

On admission a normally conscious pale man was seen Physical examination revealed widespread purpuric spots on the head extremities and trunk. There were no meningeal signs A purulent exudate was seen in the pharynx The pulse rate was 120 min and the blood pressure 60 mm Hg systolic, 40 mm Hg diastolic Heart and lung auscultation was normal. The liver was palpable two fingers below the right costal margin, the edge was blunt and painful No splenomegaly was found The urine gave a positive test for aceton and protein, the sediment contained granular and hyalin casts, 10 red blood cells and 6 white blood cells per high power field Hematocrit was 40%, white blood cell count 12,900 mm³, serum glucose 93 mg/100 ml, urea 83 mg/100 ml, serum protein 6 67 mg/100 ml, sodium 140 mEq/liter, potassium 3.8 mEq/liter, glutamic oxalacetic transaminase 40 IU, glutamic pyruvic transaminase 308 IU, lactic dehydrogenase 119 IU, creatine phosphokinase 47 IU Antistreptolysin titer 50 U, and cortisol 61 μg/100 ml LE cell preparations, antinuclear antibody tests, and blood cultures were negative Monospot reaction for infectious mononucleosis was also negative Hemostatic abnormalities including thrombocytopenia, hypofibrinogenemia, low factor VIII and elevated serum fibrin/fibrinogen degradation products (FDP) proved the existence of DIC (fig 1)

Spinal puncture yielded clear acellular fluid with normal pressure and normal glucose and protein concentrations and without bacteria. An electrocardiogram showed a sinus rhythm of 120/min, the T waves were low to slightly inverted in all leads DIC was treated with heparin, shock with plasma expanders and corticoste-

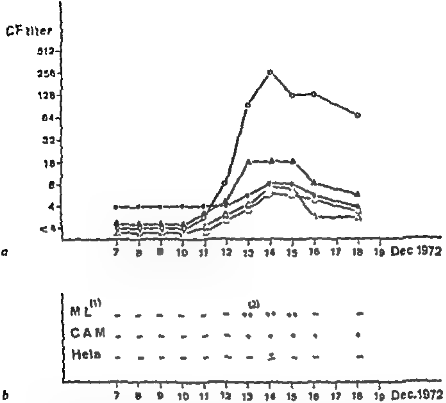

Fig 2 a Results of serological reactions (CF test) *M pneumoniae* (○) *psittacosis* (●) *adenovirus* (△) *influenza B* (▲) *influenza A* (□) b Reactivity of the patient's serum against control antigen Extracts of mice lungs (ML), chorioallantoic membranes of chicken embryo (CAM), Hela cell extract (Hela) ++ = Very strong + = strong ± = weak reaction, – = negative reaction

roids and the presumable sepsis with high dosis of ampicillin and gentamycin *On* this regimen diuresis exceeded 40 ml hourly although hypotension persisted In the afternoon a chest X ray showed pulmonary vascular congestion Intra arterial blood pressure was 65 mm Hg systolic 40 mm Hg diastolic. In the evening *inspiratory* rales were heard over both lungs The central venous pressure (CVP) was 22 cm H_2O Myocarditis was suspected and treatment with furosemide and digoxine started

The next day the CVP was lowered but hypotension persisted Isoproterenol added to the treatment increased the blood pressure progressively On December 9 1973 the isoproterenol infusion could be stopped Meanwhile respiratory failure occurred radiography of the chest disclosed diffuse infiltration of both lungs Engström ventilation was started Initially arterial pO_2 was within normal values during artificial ventilation Later on the ventilation volume and the oxygen content had to be augmented finally positive end expiratory pressure had to be added A muscle biopsy showed no evident histological signs of periarteritis nodosa.

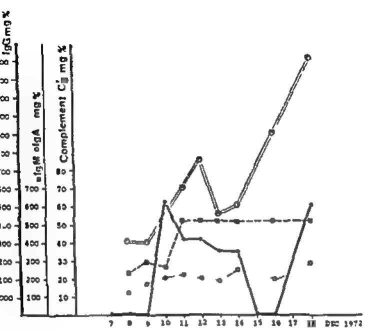

Fig 3 Results of serum IgG (□), IgM (■) IgA (○) and C'_3 (●) levels.

On December 13 the patient was neckstiff and subretinal hemorrhages were seen on fundoscopic examination Next day the hemostatic parameters pointed to reoccurrence of DIC. Finally the patient died on December 19 in irreversible shock. Diffuse hemorrhagic bronchopneumonia with interstitial pneumonitis and postnecrotic cirrhosis were found at autopsy No fibrin deposition in small blood vessels was demonstrated

Antibody titers against different antigens were measured during the course of the disease The first serum sample was obtained on December 7 and a second one on the 14th The complement fixation (CF) antibody titer against *M pneumoniae* was rising from 0 to 256 At the same time there was a slight change in the titer of some other CF antibodies (influenza A and B psittacosis adenovirus) but no difference in the antibody titer against rubella Coxsackie B 1–6 para influenza 1–4 Cold agglutinins and heterophile antibodies were absent Australia antigen was detected in the serum by means of counter immunoelectrophoresis and complement fixation Additional laboratory examinations were performed in order to investigate the multiple titer changes It was possible to situate the antibody rise between December 11 and 14 by means of CF tests on daily serum and plasma samples (fig 2)

Extracts were prepared from non infected mice lungs chorioallantoic membranes of the chicken embryo and Hela cells These were used as control antigens in a CF test The results of these tests showed a positive but transitory reaction with

extracts of mice lungs (very strong reaction) of chorioallantoic membranes (strong reaction) and of Hela cell (weak reaction) (fig 2) The *M pneumoniae* CF antigen used is not prepared on tissue culture, so we did not dispose of a suitable control antigen The results of daily serum IgG, IgM and IgA levels, C_3 concentrations are shown in figure 3

Discussion

Although there is now ample epidemiological evidence that *M pneumoniae* is an important cause of lower respiratory tract disease, fatal outcome is rare [2, 5, 9] This contributes to the concept that *M pneumoniae* infections are essentially benign Yet occasional patients experience overwhelming disease [4, 7–10] In the case presented, respiratory failure occurred in association with a significant rise of *M pneumoniae* complement fixation titer Although Mycoplasma isolation was not attempted the progressive rise to an elevated titer, about 2 weeks after the beginning of the illness, is a strong argument for the occurrence of *M pneumoniae* in this case

On admission the hemostatic parameters including thrombocytopenia hypofibrinogenemia, a low factor VIII level and elevated serum FDP values prove the occurrence of DIC During treatment with heparin a significant increase of the fibrinogen and the factor VIII levels and a decrease of serum FDP s were found To our present knowledge, clear evidence of DIC associated with *M pneumoniae* has not been reported Only one case with widespread thrombosis has been described, but without detailed blood clotting study [7] As DIC is reported in an increasing number of bacterial and viral infections [3], a causal relationship between a *M pneumoniae* infection and DIC in our patient is very suggestive

Several factors may have contributed to the fatal outcome The patient suffered of an underlying cirrhosis which could impair his defense mechanisms against infections Although it is known that a specific antibiotic therapy of *M pneumoniae* does not result in a total eradication of these microorganisms, a more benign course of the disease may be expected This patient, however, did not receive any of the antibiotics generally used for treatment of *M pneumoniae*

The recurrence of DIC may also have contributed to the fatal outcome Indeed an obvious fall in fibrinogen concentration factor VIII and platelet number as well as an increase in serum FDP s, occurred at the moment of the rise to peak titer of *M pneumoniae* CF antibodies At the same time a transitory titer rise in the CF tests for influenza, psittacosis

and adenovirus was noted This could be explained by the presence of antibodies formed by nonspecific stimulation However, antibodies reacting with antigenic material from mice lungs, chorioallantoic membranes and Hela cells were demonstrated in the serum of the patient. According to these findings an autoimmune antibody formation against antigenic material liberated from the patient's tissues can be postulated The recurrence of a consumptive coagulopathy may be due to the presence of immune complexes that activated the complement system [1, 12] The precipitous fall in C_3 level, simultaneously with the rise of specific and nonspecific antibodies, is an argument for the presence of immune complexes

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Pappenheim-Preis 1974

Der Pappenheim Preis 1974 wurde während der gemeinsamen Tagung der deutschen und österreichischen Hämatologen in Wien an Dr U R KLEEBERG Dr V KLEEBERG, Dr E BOLLING und Dr H J SEIDEL, Ulm, für die Arbeit «Metabolische und funktionelle Defekte peripherer Blutzellen bei der Leukämie» verliehen. Für hervorragende Arbeiten auf dem Gebiet der Hämatologie wird dieser wissenschaftliche Preis von der deutschen Gesellschaft für Hämatologie ausgeschrieben und von den Nordmark Werken mit einem Geldbetrag dotiert

Mitteilung der Deutschen Gesellschaft für Hämatologie

Auf der letzten Mitgliederversammlung wurde Prof Dr H D WALLER, Medizinische Universitätsklinik Tübingen, zum geschäftsführenden Vorsitzenden für die Jahre 1975–1977 gewählt Zum Kongresspräsidenten 1975 wurde Prof Dr H LÖFFLER, Medizinische Kliniken und Poliklinken der Universität Giessen, gewählt. Zum Sekretär der Gesellschaft für die Jahre 1974–1979 wurde Dr K P HELLRIEGEL, Medizinische Universitätsklinik Köln, gewählt Anschrift des Sekretariates Josef Stelzmann Strasse 9, *D-5 Köln 41*

Der nächste Kongress findet gemeinsam mit der Österreichischen Gesellschaft für Hämatologie vom 29 9 bis 1 10 1975 in Bad Nauheim unter dem Thema «Maligne Lymphome» (einschliessl der monoklonalen Gammopathien) statt

Acta Haemat. 52 129–140 (1974)

Generalized Mastocytosis

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Abstract The case histories of three patients with
:neralized mastocytosis are presented and discussed
he first patient had extensive urticaria pigmentosa,
iepatosplenomegaly polylymphadenopathy and mye
lofibrosis. The best therapeutic results were obtained
with prednisone in combination with an antihistamine
The second patient showed virtually no skin lesions The latter disappeared after
but had splenomegaly and upper abdominal pains histamine concentration
splenectomy The spleen disclosed a greatly increased that was cured by splenec
The third patient started with thrombocytopenic purpura with mast cell proliferation
tomy Seven years later urticaria pigmentosa developed to skin biopsies, determi
in skin and bone marrow and a low IgM level In addition valuable diagnostic information
nation of histamine in blood and urine can supply mastocytosis immune deficiency
especially in the more advanced cases In systemic
may occur

Key Words
Histamine
Immunological disorders
Mast cells
Mastocytosis
Urticaria pigmentosa

The first report on urticaria pigmentosa was published by NETTLESHIP [31] in 1869 The significance of mast cell proliferation in the skin in this disease was pointed out 18 years later by UNNA [44] It was not until 1949 that ELLIS [14] published the first description of generalized mast cell proliferation in human urticaria pigmentosa with reference to post-mortem findings The mast cell or mastocyte is a tissue cell which con tains metachromatically stainable granules [15] This stainability is caused for the most part by acid mucopolysaccharides such as heparin, and by histamine In human urticaria pigmentosa, the histamine from the mastocytes is of particular significance it explains many of the symptoms

Case Histories

Patient A was born in 1934. He was hospitalized in April 1969 with suspected Dichlorvos intoxication (Dichlorvos = dichlorovinyl-dimethyl phosphate, a cholinesterase inhibitor used as insecticide). A few hours after spraying with this agent in an enclosed space, the patient had had a violent attack of dyspnoea, headache, flushing and hypersalivation. Since the age of 17 he has increasingly suffered from a red, itching skin eruption described as urticaria pigmentosa. In 1964 and 1965 these symptoms had been clinically treated with antihistamines. At that time, no systemic abnormalities were found apart from a prepyloric ulcer. The patient completed a total of four courses of therapy for ulcer symptoms. In addition, he occasionally complained of vague upper abdominal pain which did not respond to a diet. There were no reports on food intolerances. Defaecation was sometimes frequent, the stools were of normal colour and substance, but sometimes slightly pulpy. In 1937 the patient had suffered from osteomyelitis of the left thigh, with a relapse in 1969. There was no family history of urticaria pigmentosa. The patient's son was suffering from asthma and other allergies.

Physical examination disclosed diffusely distributed, often confluent urticariae, many of which were pigmented. The skin showed an urticarial response to friction. There was bilateral enlargement of the retromandibular, cervical, axillary and inguinal lymph nodes. A moderately protracted expiratory sound and some bronchitis like murmurs were audible over the lungs. The liver was palpable, with a firm edge, one finger breadth below the costal arch, the spleen was also palpable one finger breadth. The left thigh showed scars due to the above mentioned osteomyelitis.

Laboratory findings: ESR 7 mm after 1 h, haemoglobin, 15.6 g/100 ml, haematocrit 44%, erythrocyte count 5,160 000/μl, reticulocytes 0.8%, leucocyte count 7500/μl, normal differential count, platelet count 320 000/μl. Routine urinalysis normal. Liver functions were normal apart from a slightly increased serum alkaline phosphatase, 155 μM min/l, it consisted of liver phosphatase. Normal basal serum calcium and phosphate values. Hypocalciuria, normal 4 hour skeletal calcium retention percentage. Total serum protein and protein pattern normal. Serum cholesterol at low limit of normal. 130 mg/100 ml.

Radiological findings: the chest X ray showed an enhanced lung pattern but no other special features. Gastrointestinal contrast radiography: coarse gastric mucosal folds, the mucosa of the deformed duodenal bulb showed a similar coarseness of folds, no other special features. The cranial X ray showed a circumscribed osteolytic focus in the right parieto-occipital area, and increased density of the sella turcica and sphenoidal sinus. Vertebral column and pelvis showed diffuse sclerosis but an intact structure. A circumscribed rarefaction was seen in the distal segment of the right femur. The left femur showed a lateral excurvation, and the middle one-third showed an enhanced bone pattern. Both femurs showed marked periosteal deposits, which were also visible on the tibiae.

Lung function tests revealed diminished vital capacity and a reduced tidal volume which increased after administration of thiazinamium (Multergan®).

Faeces: benzidine reactions negative. Normal gastric acid values: BAO 1.5 mEq/h. The fat balance showed normal fat absorption without excessive loss except on

one occasion during a period of diarrhoea 24 g fat/24 h in the faeces. Rectoscopy disclosed normal mucosal features up to 22 cm above the anal sphincter Liver scanning was indicative of hepatomegaly but otherwise normal

Histology and cytology a skin biopsy specimen contained an excess of mast cells. No mast cells were observed in a liver and a jejunal mucosa biopsy specimen. A biopsy specimen from the iliac crest showed mild myelofibrosis with slightly increased osteoclast and osteoblast activity There was an abnormally large amount of osteoid tissue with an irregular trabecular pattern and a fair number of mast cells, some of which were degranulated. A sternal bone marrow punctate contained 2% basophils, half of which were granulocytes and half tissue cells (mastocytes)

Punctates from the axillary and inguinal lymph nodes contained about 90% lymphocytes with an occasional lymphoblast, some segmented granulocytes and about 2% mastocytes A coagulation study revealed an increased prothrombin time of 15 6 sec as isolated abnormality (control value 13 1 sec) Serum immuno-electrophoresis showed a normal IgA concentration, the IgG concentration was slightly increased, while the IgM concentration showed a marked increase 5 HIAA in the urine negative on three occasions and <5 mg/l (normal) on two occasions. Serum histamine content at 10 00 h 0.230 μg/ml, at 16 00 h 0 110 μg ml (normal value <0 150 μg/ml) Urinary histamine level 900 μg/24 h (normal value 5–90 μg/24 h) The serum cholinesterase level was normal

Cholinesterase intoxication was not demonstrable Partly because it was found that the patient had had previous attacks of the skin with which he had been hospitalized, the condition was diagnosed as an attack of dyspnoea in generalized mastocytosis, possibly provoked by spraying with an insecticide. Antihistamine (Cyproheptadine®) medication was instituted, and succeeded in suppressing the itch for a few months, after which complaints about itching increased again, while the patient developed renewed brief attacks of dyspnoea, flushing and headache These symptoms diminished after a switch to another antihistamine, alimemazine, 300 mg daily

Itch exacerbated in 1971 the hepatosplenomegaly had increased, as had the superficial lymphadenopathy It was no longer possible to alleviate the symptoms with antihistamines alone An attempt was therefore made to control the itch by oral administration of [illegible] mg prednisone, later reduced to a maintenance dose of 10 mg daily The prednisone dosage could not be reduced further without subjecting the patient to an unbearable itch. During this medication the patient developed gastric symptoms which were combated by means of Alucol® (aluminium hydroxide) The findings of liver biopsy and bone biopsy did not differ from those at earlier biopsies.

In 1972 the patient reported varying attacks of pain in the area of the spleen At palpation the spleen felt nodular and extended four finger breadths below the costal margin

Early in 1973 the patient reported violent pain in the left abdomen, which was found to be d[illegible]

of [illegible]

100 [illegible]

70% [illegible]

Patient B was born in 1910 In 1932 he was rejected for navy service because of splenomegaly From 1934 on he had intermittent upper abdominal symptoms which

were treated by a porridge diet Pain in the left upper abdomen developed in 1943 In 1944 hepatosplenomegaly and slightly increased clotting time were diagnosed.

In 1958 he submitted to a course of therapy for two duodenal ulcers. In 1958 he spontaneously developed blue discolourations of the skin Examination at that time revealed hepatosplenomegaly, slight anaemia (Hb 13 4 g/100 ml) thrombocytopenia (platelet count 51,000/μl), slightly increased alkaline phosphatase, an increased prothrombin time (18 2 sec, control value 14.3 sec), and a positive Rumpel Leede test.

In 1962 the patient was admitted to our clinic with pain in the left hemithorax, unrelated to meals, efforts or emotions In addition he complained of itch and palpitations Physical examination revealed a number of elevated red spots and small vasodilations, particularly on the trunk The liver was palpable over two finger breadths, the spleen was palpable almost two hand widths below the costal margin on the left, no other abnormalities

Laboratory findings ESR 8 mm, haemoglobin 13 6 g/100 ml, haematocrit 38%, erythrocyte count 4 6 *million/μl, reticulocytes* 1 4%, leucocyte count 4,500/μl differential count normal, platelet count 50 000–100 000/μl Routine urinalysis no abnormalities Faeces benzidine reactions negative Normal serum urea creatinine and fasting glucos levels, normal liver function tests apart from an increased alkaline phosphatase value (200 μM/min/l) Total serum protein and protein pattern normal

Radiological *findings* the chest X ray showed an enhanced bilateral lung pattern, no other abnormalities Spleen shadow extended down to 15 cm below the left costal margin Skull, upper arms forearms, legs and thighs without abnormalities. IVP no abnormalities normal excretion of contrast medium

Histology and cytology numerous mast cells were found in a skin biopsy The highly cellular *sternal punctate showed 3% mastocytes*, but no other abnormalities. A lymph node biopsy showed a normal structure, but many mastocytes stained metachromatically with toluidine blue In the liver punctate, normal structures were found, in the periportal triangles cells with metachromatically staining granules, i e mast cells A bone biopsy specimen showed a normal structure, but again with a *large* number of mast cells A spleen punctate contained about 75% lymphatic cells, 10% segmented cells, *monocytes*, and 10–12% basophile tissue cells

The serum histamine level was 0 05 and 0 13 μg/ml (normal value 0 150 μg/ml) A limited coagulation study revealed no abnormalities apart from moderate thrombocytopenia and a slightly increased prothrombin time

After taking a biopsy specimen from the skin lesions the patient s condition was initially diagnosed as cutaneous angiomatosis in view of the phlebectasiae in the skin Once metachromatically stainable basophile tissue cells had been demonstrated in the skin the diagnosis was altered to telangiectasia macularis eruptiva perstans a rare form of urticaria pigmentosa with generalized mastocytosis Antihistamine medication was instituted which alleviated the pain as well as the itch

In January 1963 the patient was readmitted with symptoms possibly indicative of hypersplenism thrombocytopenia and slight anaemia, with highly cellular bone marrow specimens showing no abnormalities apart from slight mastocytosis. Partly because it was assumed that the symptoms would diminish after removal of an organ containing numerous mastocytes, a splenectomy was performed A spleen weigh

ing 1,610 g was removed The capsule of this very large spleen was thickened Microscopic examination disclosed numerous accumulations of mast cells in the red pulp, particularly along the trabeculae The white pulp contained few mast cells The histamine content of the spleen tissue was 570 μg/g tissue (normal value <50 μg/g) The post-operative course was uneventful, and the patient's condition gradually improved Pain and itch disappeared The haemoglobin value returned to normal The platelet count rose to 900 000/μl shortly after the operation, but eventually returned to normal Serum and urinary histamine levels continued to fluctuate, showing sometimes normal, sometimes slightly increased values In the course of the years the patient occasionally reported vague upper abdominal symptoms, based on a gastritis (radiologically deformed duodenal bulb) Basic gastric acid values were normal (BAO 2.1 mEq/l) Palpitations were less frequent than prior to the operation At a recent follow up (June 1974) the patient was free from symptoms A bone marrow punctate (1970) showed similar features to that of 1965 a slight increase in basophile tissue cells to about 4% of the nucleated cells.

Patient C was born in 1939 In 1961 appendectomy was performed. In 1964 he was admitted for idiopathic thrombocytopenic purpura, without itching At physical examination many petechial spots and haemorrhages of the oral mucous membranes were seen. No lymph nodes could be palpated Laboratory data revealed a low platelet count (0–9,000/μl) and normal values of Hb, Ht and leucocyte differential count. The highly cellular sternal bone marrow punctate showed normal features with megakaryocytes in normal numbers. Antibodies directed against nuclear proteins could not be demonstrated Treatment with high doses prednisone was started, without any influence on platelet count. So splenectomy was performed The spleen had a normal macroscopic and microscopic appearance No histamine content was estimated, as no mastocytosis was expected Post-operatively the platelet count rose to normal values

In 1969 nephrectomy on the right side was performed for recurrent urinary tract infections with colics Pathological diagnosis of the small kidney (weight 130 g) was nephrolithiasis in chronic pyelonephritis. No abnormalities in the calcium metabolism could be found (normal basal serum calcium levels and calciuria)

In the beginning of 1971 the patient discovered some browny spots, with a red appearance on mechanical irritation on this trunk. No itching was experienced Physical examination revealed no other abnormalities than the browny pigmented spots on the skin of the trunk.

Laboratory findings ESR 4 mm, haemoglobin 15.6 g%, haematocrit 47.2%, erythrocyte count 4.37 million/μl, reticulocyte count 0.9%, leucocyte count 7,000/μl with normal differential, platelet count 546 000/μl Routine urinalysis no abnormalities. Serum urea 0.5 g/l, creatinine [illegible]1 mg/l (slightly increased), normal liver function tests, total serum protein 77 g/l, with a slightly increased γ-globulin

Radiological findings no abnormalities on the chest X ray Gastrointestinal contrast radiography also normal X rays of the skull, vertebral column and pelvis were normal, *but both tibiae showed marked periostal deposits*

Histology and cytology numerous mast cells were found in a skin biopsy specimen. The sternal bone marrow punctate showed 1–2% mastocytes, but no other abnormalities A needle biopsy of the liver was normal, no mast cells were found

Serum immunoelectrophoresis showed a normal IgA concentration (166 mg/

100 ml) The IgG concentration was increased (2 170 mg/100 ml), while the IgM concentration was very low, 12 mg/100 ml 5 HIAA in the urine was normal (5 mg/l), the urinary histamine excretion on three consecutive days was also normal with a mean value of 38 µg/24 h Faeces benzidine reactions negative Normal fat excretion and normal urobilinogen excretion with the faeces Gastric acid values were high (basic gastric acid output 9 25 mEq/h)

Diagnosis urticaria pigmentosa with some systemic involvement As the patient had no complaints no symptomatic treatment was instituted At a recent follow up (November 1973) the cutaneous symptoms were identical, as were the haematological data

Discussion

Mastocytes are cells of the RES, and normally occur in small numbers in all organs They possibly play a role in the reaction of the organism to noxious stimuli Increased numbers of mastocytes have been observed in the skin in urticaria pigmentosa and basal cell carcinoma [25], psoriasis and other skin diseases [35] In urticaria pigmentosa, however, larger accumulations of mastocytes are encountered Increased numbers of mastocytes have also been observed in callus tissue [41, 47] The dermatological abnormalities of urticaria pigmentosa can be present at birth or occur shortly after The most common findings being yellow-brown pigmented spots or specks which show an urticarial reaction to friction and in that case frequently cause an itch The skin affection can be confined to a single site ('mastocytoma') but usually shows diffuse spread over the entire body Macroscopic features include pigmented urticariae (urticaria pigmentosa) or brown-pink spots with delicate telangiectases (telangiectasia macularis eruptiva perstans [27]) The condition may be associated with a positive dermographia and urtication of the lesion after mechanic irritation (Darier's sign) [36]

The aetiology of mastocytosis is obscure Several authors assume that it is reticulosis [2, 18, 22, 34] In this respect immunological factors may be important This can be manifested in an abnormal immunoglobin pattern [10, 26 31, 38, 45] as is illustrated by patient C, or an abnormal protein pattern at paper electrophoresis, as we found in patient A Perhaps these immunologic abnormalities will correlate with systemic spread of the mast cells

The principal localizations of generalized mastocytosis are the skin, bone marrow, lymph nodes, liver and spleen In patient A the cutaneous symptoms paralleled the other symptoms The latter were probably

caused by histamine that was demonstrated in increased amounts in blood and urine. However, the severity of symptoms cannot be correlated to the absolute value of the histamine level. It is true, meanwhile, that an increased blood histamine level is often accompanied by aggravation of symptoms [12, 35]. It is not always possible to demonstrate mastocytes in a biopsy specimen, because without special staining it is difficult to identify these cells [5, 6, 8]. In the absence of immediate biopsy evidence of generalized mastocytosis, despite abnormally high histamine levels in blood and urine, the diagnosis of generalized mastocytosis is nevertheless to be considered, particularly if there are typical skin changes.

The respiratory disorder, which is suggestive of asthma, probably has to do with the increased histamine level in the blood. The enhanced lung pattern on radiographs probably results from infiltration in the lung by the cells; this has been repeatedly confirmed at post mortem examination [30, 43]. The enlargement of lymph nodes, liver and spleen is to be chiefly ascribed to mast cell proliferation, but fibrosis can also play a role [6, 16]. This was confirmed at microscopic examination of the spleen from patient B.

Substances other than histamine can also cause flushing and positive dermographia. The best known of these substances is serotonin, the level of which is increased in the carcinoid syndrome. In patients showing flushing, a search is often made for metabolites of serotonin (5-hydroxyindole-acetic acid). An increased 5-HIAA excretion has sporadically been demonstrated in mastocytosis. Another possibility is to be found in kinins such as bradykinin, which can cause urticaria and vesiculation. WINKELMAN *et al.* [47] demonstrated increased kinin activity in the skin in urticaria pigmentosa. Moreover, the reaction of the tissues to these substances can be changed in urticaria pigmentosa [23]. MOORE ROBINSON and WARREN [29] demonstrated a changed reaction of the skin to histamine in urticaria pigmentosa after administration of salicylates. Gastrointestinal symptoms can be largely interpreted as histamine effects, e.g. diarrhoea as a result of accelerated intestinal peristalsis. Another cause of diarrhoea may be diminished absorption as a result of diffuse mast cell infiltrates in the intestinal wall [18, 22] or atrophy of the villi in the small intestine [1]. Although the case history often makes mention of ulcer symptoms, normal gastric acid values are frequently found. Both our patients had suffered from gastric or duodenal ulcer, and both showed normal gastric acid values. Their ulcers may have been stress ulcers [43].

Biochemical changes such as increased serum alkaline phosphatase are

probably due to slight disorders of liver function, as does a somewhat low serum cholesterol level [34]. The low cholesterol level is sometimes related to hyperheparinaemia [17]. This might explain the coagulation disorders also [21]. However, an increased prothrombin time can also result from disturbed liver functions. A low platelet count could suggest hypersplenism, as it did in patients B and C, whose platelet counts returned to normal after splenectomy. A correlation between the thrombocytopenic purpura (patient C) and mastocytosis could not be confirmed by histological analysis of the spleen. But as no suspicion on mastocytosis was raised at the operation no special staining was possible.

Patient A showed an osteoporotic focus in the skull as well as osteosclerosis of the sphenoid bone. Other skeletal parts chiefly showed osteosclerosis. Generalized osteoporosis can be related to diarrhoea and diminished calcium absorption [42]. A biopsy specimen from patient A was suggestive of myelofibrosis with mast cell proliferation. No distinct radiological changes were found in patient B and only small periosteal deposits in patient C. In all patients the bone marrow contained too many mast cells. Anaemia, if it exists, can be due to somewhat hypoplastic myelofibrosis [6] or to hypersplenism. Both osteoporosis and myelofibrosis can result from mast cell proliferation, heparin is believed to play an important role in this respect [13].

The prognosis of generalized mastocytosis is uncertain. Generally speaking, it needs not shorten the life-span. It is a common condition in dogs. Cook [9] described a post-mortem study of dogs which had died from natural causes, he found the mean life-span of dogs in which mastocytosis was diagnosed to be in fact slightly longer than that of dogs without mastocytosis. Of course this is not necessarily applicable to man, but it does suggest that generalized mastocytosis need not *a priori* be malignant. The risk of an early death is increased in the presence of complications such as cachexia, anaemia, intractable diarrhoea, haemorrhages or shock [30]. In a number of cases, mastocytosis degenerates to leukaemia, basophile tissue cells entering the bloodstream [6, 11, 37, 40]. Marantz and Roth [26] described a patient with long-standing urticaria pigmentosa who ultimately died from the complications of a malignant lymphoma. In patient A, only basophile granulocytes were found in the peripheral blood, but the entire course of his illness raises the fear of malignant degeneration in future.

Therapeutic possibilities in cutaneous as well as in generalized mastocytosis are limited. No causal therapy is known. In view of the fact that

mastocytes contain several substances (histamine, heparin, serotonin) which can be held responsible for the symptoms, medication has primarily focused on these substances In a number of cases it proved possible to reduce the symptoms with the aid of antihistamines As a rule, however, it is impossible to effect complete disappearance of symptoms, and in the course of time, the condition becomes refractory to the agent used, necessitating a switch to a different antihistamine In this respect it is important to start with a sufficiently large dose, lest any perceptible therapeutic result will remain absent Other antagonistic agents have been used with varying success, e g histidine decarboxylase inhibitor [4, 32], a serotonin and histamine antagonist [28], reserpine [12, 20] and L-hyoscyamine [5] Protamine sulphate seems useful only if there is a distinctly demonstrable heparin effect in the circulation, expressed in a markedly increased clotting time [22] Apart from antagonists, therapeutic attempts have been made with agents which degranulate mast cells The best results have been recorded with corticosteroids The results obtained are variable [19, 24, 39] but particularly short term results are sometimes very promising [1]

X-ray irradiation also causes degranulation of mast cells This seems to have a favourable effect, particularly in local application to combat, say, deep-seated ostealgia [43] But for treatment of the entire body the dose required is so large that side effects become predominant Cytostatic drugs have been used with varying success in the case of leukaemic degeneration [11, 40] These results are comparable to the variable results generally obtained with cytostatics in the treatment of immature cell leukaemias Analogous to therapeutic considerations in idiopathic histiocytosis [46], cytostatic medication can also be considered in generalized mastocytosis, even before any sign of malignant degeneration is evident Surgical treatment of mastocytosis seems indicated only in cases which involve an isolated mastocytoma [3, 7] with systemic symptoms Splenectomy is to be considered in the case of hypersplenism The condition of patient B clearly improved following splenectomy, but it is difficult to establish whether this remission was exclusively due to the operation or involved 'spontaneous improvement as well The urticaria pigmentosa of patient C did not seem to be influenced by the splenectomy

Patients with mastocytosis can show intolerance to acetosal and codeine, which only serve to aggravate their symptoms This is probably a result of a degranulating effect of these agents on the mast cells A similar reaction may be observed after consumption of alcohol and certain herbs

Patient A cannot tolerate acetosal or codeine but shows no intolerance to alcohol. Patient B and C show no sign of any intolerance. Physical and mechanical irritation of the skin in urticaria pigmentosa can provoke skin symptoms. Patient A discovered that firm brushing of his entire body surface initially aggravates the itch, but after a while alleviates it to a substantial degree. He has therefore accustomed himself to this ritual shortly before turning in and consequently spends a more comfortable night (with less itching) than without this brush treatment.

Patients with cutaneous mastocytosis should be examined for any possible systemic spread of the mast cell proliferation. In the follow-up this examination should be repeated at certain intervals so that any such spread can be identified as early as possible and dealt with. Determination of the presence of histamine in blood and/or urine can be a valuable diagnostic aid in this respect.

Acknowledgements. For help and advice I thank S. A. Duursma, MD, Prof. L. W. J. Holleman, Ph. D., W. Sizoo, MD, J. Ph. H. B. Sybesma, MD and W. J. Visser, Ph. D.

I deeply regret the death of Prof. M. C. Verloop, MD, who gave me the idea to write this study.

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Acta Haemat. 52 141–150 (1974)

Effect of Starvation on the Response to Erythropoietin in the Rat[1]

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Abstract. The response to ESF has been studied in normal, starved, polycythemic, and starved polycythemic rats. Starvation markedly reduces this response in normal and polycythemic rats. Disappearance rate of ESF is normal and no inhibitor is detectable in the plasma of starved rats. Experimental data suggest that starvation decreases the ESF-sensitive stem cell pool.

Key Words
Erythropoietin
Polycythemia
Rat erythropoiesis
Starvation

Depression of erythropoiesis is a well-known effect of starvation [1, 2]. From the observation that erythropoiesis can be restored to normal levels by erythropoietin (ESF), it has been inferred that the responsiveness to the hormone is not affected by starvation, and it is generally admitted that a decreased production of erythropoietin is responsible for the depression of erythropoiesis [2]. However, administration of high doses of ESF could compensate a decreased responsiveness to the hormone and restore erythropoiesis to normal levels. Actually, most of the studies reported concern protein deprivation [3, 4], and it has not been unequivocally established, in fact, that ESF responsiveness is normal in starvation. As we had occasionally observed a decreased response to ESF in the

[1] This work was performed under contract Euratom ULB Pise No 063-4 BIAC and supported by a grant of FRSM (Belgium).

[2] The authors thank Mrs. M. de Hovre and M. [illegible] Parisse for their technical assistance in performing these studies.

starved rat, we attempted to investigate the subject more thoroughly in this study

Material and Methods

Effect of ESF on erythropoiesis in the starved rat. Normal and polycythemic male Wistar rats (180–200 g) have been used in experimental groups of four animals. Polycythemia has been achieved by exposure to simulated altitude during 3 weeks (320 mm Hg, 8 h/day). In one experiment, transfused polycythemic rats have been used (2 × 7 ml red cells, Hct 75% i.p.). Four days after arrest of hypoxia or the second transfusion, starvation was started (day 0). ESF was injected i.v. or s.c. on day 1, or on day 1 and 2. Radioiron (0.5 μCi ^{59}Fe citrate) was injected in a tail vein on day 3. Incorporation into red cells was measured on day 4, 22 h later. Blood was obtained by heart puncture, red cells were washed twice in saline before counting, and incorporation was calculated estimating the blood volume to be 5% body weight in the normal and 7% in the polycythemic rats, respectively. To obviate dehydration resulting from water refusal during starvation, polycythemic starved as well as normal starved rats were injected i.p. with 10 ml saline on day 1, 2 and 3 except in one experiment (table I, exp. 1). ESF was obtained from urine of an anemic patient according to the method of Lowry and Borsook [5], 2 mg lyophilized extract being equivalent to 1 unit IRP.[a]

Effect of starvation on the disappearance rate of ESF. Normal controls and rats starved for 65 h were injected i.v. with 2 ml active plasma (ESF titre ± 4 U/ml) obtained from hypoxic rats of the same strain, killed immediately after hypoxia. Rats were killed 0, 1, 2, 4 and 6 h after the injection of active plasma. ESF assays were performed on the pooled plasma of 4 rats/group by a modification of the method of De Gowin *et al.* [6] using transfused posthypoxic mice. Six virgin female TO mice weighing 20–25 g were used for each assay. Mice were injected with 1 mg iron (Imferon) before exposure to hypoxia (3 weeks at 320 mm Hg). 4–5 days after removal from the altitude chamber the mice were injected with 1 ml blood i.p. (Htc 75%). Five days after the transfusion they were injected subcutaneously with 1 ml plasma. Two days later radioiron was injected via a tail vein (0.5 μCi ^{59}Fe Cl_3) and 3 days afterward incorporation of radioiron into red cells was measured, assuming a blood volume of 7% body weight. Results have been expressed in units by reference to a dose-response curve constructed with standard IRP. The regression curves have been calculated by the least squares method, considering as 100% the values of ESF titer at 0 time immediately after the injection.

Serum iron was measured with an autoanalyzer by the method of Young and Hicks [7]. Hematocrit was measured by a micromethod on the blood obtained by heart puncture. Reticulocytes were counted by the direct smear method using brilliant cresyl blue, as percent of 1000 cells. An index was chosen to express the results by a number proportional to the absolute amount of reticulocytes, corresponding to the product of the calculated red cell mass in milliliters, by the number of reticulocytes per thousand.

[a] International reference preparation provided by the Department of Biological Standards of the National Institute of Medical Research, London.

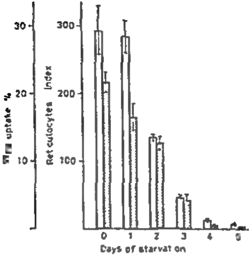

Fig. 1. Effect of starvation on erythropoiesis of the normal rat measured by ^{59}Fe incorporation into red cells (open columns) and reticulocyte index (hatched columns) (vertical bars = mean ± SE).

Results

Effect of Starvation on the ^{59}Fe Incorporation into Red Cells and on Reticulocytosis

Starvation decreased erythropoiesis very rapidly, as shown in figure 1. The incorporation of ^{59}Fe into red cells decreased from 29.51 to 0.54% after 5 days and the reticulocyte index from 217 on day 0, to 3 on day 5 of starvation.

Effect of Starvation on the Response to ESF

Compared effects of a single intravenous and two subcutaneous doses of ESF (table I). The response to ESF whatever its mode of administration, either in a single intravenous or in two subcutaneous doses on two consecutive days was markedly reduced in the starved groups. The response to 1 U / 2 s. c. of polycythemic fed rats was higher than the response to 2 U / 2 s. c. of starved nonpolycythemic animals. Iron uptake was hardly increased in polycythemic starved rats. Results were similar in experiment 1 and 2 despite parenteral rehydration of the starved groups with 10 ml saline on days 1, 2 and 3 in experiment 2.

Table I Effect of starvation and polycythemia on ^{59}Fe incorporation into red cells (mean ±SE)

	Hematocrit, %	^{59}Fe incorporation			
		saline	2 U ESF	1 U ESF	2 U ESF
			i v 1X	s c 2X	s c 2X
Experiment 1					
Starvation	53±0.5	5.05±1.07	10.91±0.20	18.45±1.80	26.84±3.0
Polycythemia	66±1.0	2.75±0.65	18.15±3.31	35.28±2.42	–
Polycythemia +starvation	79±1.0	0.20±0.02	0.53±0.06	4.58±1.72	5.26±1.53
Experiment 2					
Starvation	49±1.0	4.77±1.35	9.91±1.75	13.24±4.60	25.75±1.90
Polycythemia	62±1.0	2.77±0.28	22.96±0.96	30.30±2.05	–
Polycythemia +starvation	70±1.0	0.49±0.04	2.38±0.33	6.98±0.85	9.85±1.86

In three experimental groups, serum iron determinations were performed 2 days after a single injection i v of ESF Values of serum iron in normal starved, polycythemic starved, and polycythemic fed rats, were 128 (SEM ± 23), 155 (SEM ± 5), and 236 (SEM ± 19) μg%, respectively

Effect of increasing doses of intravenous ESF In these experiments increasing doses of ESF (1, 2, 3 and 5 U) were given i v in one single injection Additionally, a total dose of 3 U was given in two divided doses on two consecutive days In fed polycythemic rats, a log-dose relationship between the dose of ESF and the incorporation of ^{59}Fe into red cells was observed In starved animals the response to increasing doses was less clearly defined, only a slight elevation being observed between 1 and 5 U If 3 units of ESF was injected in two doses, the response of polycythemic fed rats was also higher than in starved controls, the Fe uptake increasing to 29% in the polycythemic rats, and to 12% in the starved normal rats In the polycythemic starved groups a better response was also observed after two divided doses The reticulocyte index followed a similar pattern (fig 2) The same results were obtained when transfused instead of hypoxic polycythemic rats were used A single i v injection of 3 U ESF increased the uptake of starved and fed transfused rats to 7.5 and 27.4%, respectively, and the reticulocyte index to 22 and 186 (fig 3)

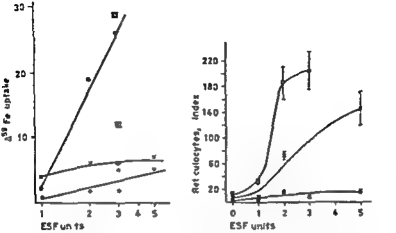

Fig 2 Erythropoietic response measured by Δ ^{59}Fe uptake (^{59}Fe uptake minus saline) and reticulocyte index (mean ± SE) of polycythemic (○) polycythemic starved (●) rats and starved controls (×) to increasing doses of ESF Two divided doses same symbols in squares

Table II Incubation of ESF rich plasma (2.5 ml) with starved or polycythemic rat plasma

ESF rich plasma +	Normal plasma	Polycythemic plasma	3-day starved normal plasma	3-day starved polycythemic plasma
Experiment 1	7 77 ± 1 34	8 92 ± 1.53	14 12 ± 2.13	8 13 ± 1.53
Experiment 2	11.32 ± 2 61	8 90 ± 1 80	12 74 ± 3 84	10 04 ± 2.48

Mean ^{59}Fe uptake in polycythemic mice ± SE

Effect of starvation on the disappearance rate of exogenous ESF Disappearance rate has been studied four times in normal rats, and twice in rats starved for 65 h Erythropoietin titer averaged 1 6 U/ml in controls and 1 8 U in starved rats immediately after the injection of the active plasma and was considered as 100% Results were expressed in percentage of this value As shown in figure 4, the T/2 of erythropoietin in the plasma was 1 6 h in normal rats, and a similar value was observed after a starvation period of 65 h

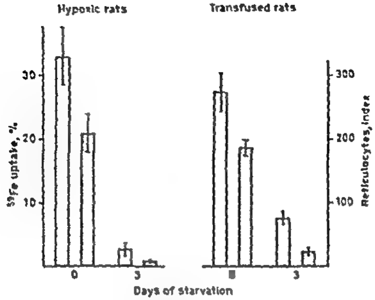

Fig. 3. Effect of starvation on the response of hypoxic or transfused polycythemic rats to a single dose of ESF (3 U i.v.), measured by ^{59}Fe incorporation into red cells (open columns) and reticulocyte index (hatched columns) (vertical bars: mean ± SE).

Search for an ESF inhibitor in starved and polycythemic rat plasma. 2.5 ml active plasma (1.2 U/ml in experiment 1 and 1.8 U/ml in experiment 2) obtained from normal rats submitted for 14 h to simulated altitude (280 mm Hg), were incubated and gently agitated in a 37 °C water bath for 1 h, with 5 ml plasma of polycythemic, normal starved or starved polycythemic rats. The incubates were assayed on polycythemic hypoxic mice. As shown in table II, there was no detectable inhibitor in the different plasmas.

Discussion

Depression of erythropoiesis by starvation in the rat is a well-documented phenomenon [1, 2], illustrated in this study by figure 1. This depression could be imputable either to a decreased production of endogenous ESF, to a reduced response of the marrow to the hormone, or to a combination of both mechanisms. The erythroid hypoplasia observed in the starved rat is generally attributed to a decreased production of ESF in relation to diminished oxygen requirements [2]. As normal levels and ac-

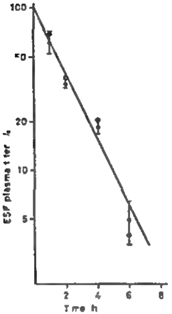

Fig 4 Disappearance rate of exogenous ESF in normal (● mean ± SE, four experiments) and fasted (○ mean of two experiments) rats

cordingly infranormal levels of ESF are undetectable by the bioassay used this view has not been unequivocally established An indirect argument in agreement with this hypothesis is the lower ESF response to hypoxia of starved rats as compared to normal controls [8] This reduced responsiveness could also be attributed to the absence of some indispensable material or to an enzymatic dysfunction of synthesis of ESF, as to a decreased oxygen demand The former mechanism seems unlikely, as ESF production is susceptible to be increased despite starvation if a more

ESF i v induced a lesser response in the starved than in the polycythemic rat, and an almost nul response in the polycythemic starved animal Results were similar whether polycythemia was achieved by hypoxia or by transfusion. The values of iron incorporation were related to erythro-

poietic activity and not to modifications of the labile iron pool the highest levels of serum iron being found in the groups with the highest values of iron incorporation Moreover, a striking parallelism between reticulocytosis and iron incorporation was observed Although the i v administration of increasing single doses of ESF induced a log-dose response in polycythemic rats, a plateau was reached in starved polycythemic and starved normal rats (fig 2) This response to a single dose of ESF could account for the number of stem cells sensitive to the hormone in the marrow, or for a deficiency of some hypothetical substance necessary to hemoglobin synthesis This latter hypothesis is quite improbable as levels as high as 27% of $_{59}$Fe incorporation could be attained in the starved rat if ESF was injected s c on two consecutive days This fact implicates that intensive synthesis of hemoglobin is possible in starvation Moreover, the response to a given dose of ESF was higher if the hormone was injected i v in two divided doses than in a single dose (fig 2) It seems, consequently, that the slight response to a single i v injection is not due to the lack of a substance necessary for hemoglobin synthesis but more likely to a reduced pool of ESF sensitive cells The increased response to divided doses of ESF suggests that recruitment of ESF sensitive cells as described by SCHOOLEY [9] is still present in the starved rat An alternative hypothesis would be a faster catabolic removal of ESF after starvation hence the lesser response to the hormone However, we have shown previously for endogenous ESF [10] and in this study, for exogenous ESF that the T/2 of the hormone was similar in starved and in normal rats (fig 4) An inhibitor of ESF has been reported in the plasma of starved rats [11] We have been unable to demonstrate any inhibitory activity in the plasma of polycythemic fed and starved normal or starved polycythemic rats GARCIA and VAN DYKE [12] observed that the response to ESF was identical in normal controls starved and hypophysectomized rats However it must be emphasized that the comparison of normal with starved rats is invalidated by the fact that erythropoiesis is studied at different levels of the dose reponse curve in normal rats the uptakes have been studied near the plateau of the curve (between 35 and 60%) whereas in hypophysectomized and starved rats the ascending linear part of the curve (between 15 and 50%) is used Consequently the signification of similar or even slightly superior responses of starved against normal rats is not evident. Our study shows clearly that starvation reduces the sensitivity of polycythemic rats to erythropoietin As starved polycythemic rats refuse water higher hematocrits are observed in these

groups However, the elevation of the hematocrit in itself does not seem to be responsible for the reduction of responsiveness to ESF since no increased response is observed if the hematocrit of starved polycythemic and starved control rats is lowered by daily saline injections (table I) As the base line of erythropoiesis in normal starved rats was higher than in normal polycythemic rats it is unlikely that the lesser responsiveness of the former group should be imputable to a lower rate of cellular recruitment. On the other hand it seems probable that in starved polycythemic rats, the marked depression of the basal erythropoiesis by polycythemia accentuates the decrease of ESF responsiveness due to starvation However it must be pointed out that in spite of similar depression of erythropoiesis, the protein-deprived rat, contrarily to the starved rat, responds normally to ESF [4]

It can be concluded that starvation decreases the response to ESF in the rat This effect seems to be related to a decrease of the ESF-sensitive stem cell pool The depression of erythropoiesis during starvation thus depends on a reduced sensitivity to ESF, possibly associated with decreased production of the hormone as has been claimed previously

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Acta Haemat. 52: 151–160 (1974)

Differences in the Intensity of ⁵⁹Fe Incorporation into Various Regions of Bone Marrow of C57BL/10 Mice after Acute Radiation Exposure

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Key Words
Bone marrow irradiation
Erythropoiesis
Iron incorporation in bone marrow
Mouse bone marrow

Abstract. The study reports on the ^{59}Fe incorporation in the marrow of various bones of mice under 'normal' conditions and after acute sublethal whole body irradiation with X- and γ-rays. The decrease of incorporation varies for the different regions of the skeleton in the first few days after irradiation. The total incorporation in different skeletal parts exhibits statistically significant changes when compared with the pre-irradiation stage. A smaller decrease may be observed in the short spongiose bones; the greatest decrease was found in the femur, humerus, tibia/fibula and pelvis. From the practical point of view, it is important that according to post-irradiation ^{59}Fe incorporation, the femur and tibia, currently used in experiments, are the most 'reactive' bones. This should be taken into account when extrapolating data obtained from these bones to the whole bone marrow of a mouse.

Incorporation of ^{59}Fe into the erythropoietic tissue of man and animals is one of the methods currently employed in the study of erythropoiesis. For technical reasons, in experiments using small laboratory animals, the long bones, predominantly the femur and tibia, are mostly used. Where a knowledge of the intensity of ^{59}Fe incorporation into the marrow of the whole skeleton is required, the above-mentioned bones are considered as representative and their activities are usually multiplied by constant coefficients. Data regarding the relative rate of ^{59}Fe incorporation into various regions of bone marrow of the mouse, which is now becoming more frequently an object of haematological studies, is still rare and/or too general [1–3] and mostly limited to the study of undisturbed erythropoiesis. In radiation haematology, the course of the de-

population and recovery of erythroid tissue after acute irradiation has been already repeatedly investigated by means of ^{59}Fe incorporation. However, the changes were usually studied on a single bone only. The present contribution is mainly focused on the examination of the possible differences in the intensity of ^{59}Fe incorporation into the marrow of various parts of the skeleton after acute irradiation with ionizing rays. Concomitantly, information was obtained on the relative intensity of ^{59}Fe incorporation into the marrow of various skeletal parts of mice kept under "normal" conditions.

Material and Methods

Male C57BL/10 mice, inbred bred, approximately 13 weeks old, were used. After 1 week of adaptation to laboratory conditions, the animals were irradiated with 100, 300, or 500 r X-ray exposure; one group of animals received 500 rad γ-radiation. X-irradiation was carried out with TUR apparatus operating at 180 kV, 15 mA, filtration 0.5 mm Cu, 0.5 mm Al, focal distance 38 cm, exposure dose rate about 60 r/min in air (measured with a Victoreen dosimeter). Groups of 5 animals were always irradiated simultaneously in a glass container. For γ-irradiation an underwater ^{60}Co 2,000 Ci source was used (apparatus manufactured by Chirana, Brno, for description and parameters see CHMELÍŘ and HOLEČEK [4]). The dose rate measured in the place of irradiation was 25.9 rad/min (measurements performed by Frick ferro-ferri dosimeter).

Parallel to each irradiated group a non-irradiated control group of the same size was examined. At appropriate intervals after irradiation, at which minima and maxima of the incorporation activity curves were expected according to our previous findings, the intensity of Fe incorporation into the bone marrow was investigated in groups of 10 irradiated and 10 non-irradiated animals. ^{59}Fe citrate (Messrs. Rotop, GDR) supplied in an isotonic buffer solution at pH 5–6, was diluted, to give an activity per mouse of 0.5 μCi/0.2 ml (specific activity 10–20 μCi/μg Fe). The solution was injected i.p. at about 8 a.m., a similar time as for the radiation exposure. Six hours later, the animals were killed by cervical spinal fracture. Freed from skin and viscera, the carcasses were placed in a colony of the insect Dermestes vulpinus kept under laboratory conditions. Exploiting the saprophagic activity of this insect, animal skeletons perfectly free of soft tissue were obtained. The skeletons were dissected int[illegible] long bones, or groups of them (tab. I), the [illegible] ities of which we[illegible] individually against corresponding sta[illegible] spectrometer N[illegible] Mark I. Paired bones of extremities [illegible] simultaneously.

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ular days after irradiation were calculated (1) as percent of injected activity, and (2) as percent of total skeletal activity of the individual mice (the latter approach reduced the interference of 'occasional' variation of total erythropoiesis on different days and between animals). Finally, the relation of values of irradiated animals to those of controls was expressed as a percentage (i. e. average values 1 and 2 of the irradiated groups as a percentage of the respective values of the control animals).

Approximately 33% of Fe incorporated into the skull during a 6-hour period may be deposited in teeth [1], so 33% of the skull activity established for a corresponding group of non-irradiated animals was subtracted from the activity of the skulls of all animals.

Results

The degree of participation of individual bones, or groups of bones, to the total ^{59}Fe incorporation into the skeletons of non-irradiated mice was calculated using all 177 control non-irradiated animals. The results are summarized in table I.

The course of incorporation of ^{59}Fe into the marrow of various skeletal parts (on the 1st, 2nd, 7th to 9th, 17th, 18th and 20th days) after 300 r X-ray exposure, calculated as percent of injected activity (calculation 1, see Methods), is shown in figure 1. The different behaviour of various bones become clearer, if incorporation of ^{59}Fe is expressed as a percentage of the total skeletal activity of individual mice (calculation 2). The values thus calculated were related to control values (%) and

Table I. ^{59}Fe incorporation in the various regions of bone marrow as percentage of the total skeletal activity (± SE) in control non-irradiated animals (n = 177)

Skull (the activity pertaining to teeth being subtracted)	7.83 ± 0.09
Vertebral column	31.11 ± 0.14
Sternum	4.35 ± 0.05
Ribs	8.93 ± 0.12
Scapula (2)	2.92 ± 0.03
Clavicle (2)	0.65 ± 0.01
Humerus (2)	6.20 ± 0.06
Radius, ulna, forefoot (2)	1.53 ± 0.03
Pelvis	13.57 ± 0.12
Femur (2)	13.54 ± 0.11
Tibia, fibula (2)	8.64 ± 0.08
Hind foot (2)	0.73 ± 0.03

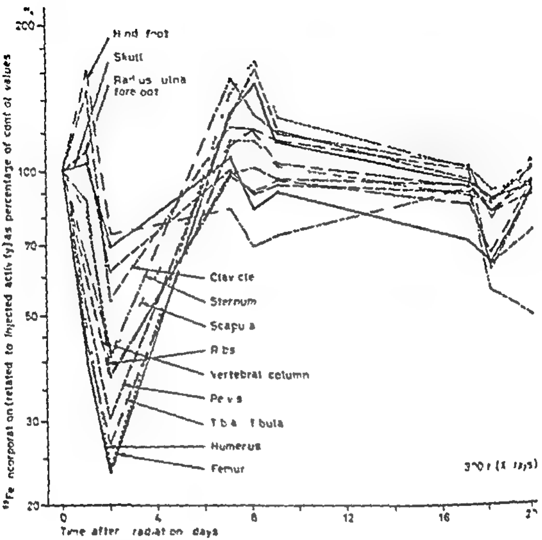


Fig 1 Incorporation of ^{59}Fe (related to injected activity) into the marrow of individual skeletal parts at various time intervals after X irradiation with 300 r exposure expressed as percentage of uptake in non irradiated mice

given with SE in table II A high statistical significance of the differences between individual skeletal regions follows from this table

The effect of irradiation of mice rad γ-rays on ^{59}Fe incorporation on the 2nd and 8th days is shown in figure 2 As sho on with order of the decrease which the 2nd day , the 8th day after

Table II Incorporation of ^{59}Fe (related to the total skeletal activity of the individual mice) into the marrow of various skeletal parts at different time intervals after X irradiation with 300 r exposure, expressed as percentage uptake in *non* irradiated mice (± SE)

Skeletal part	Days after irradiation							
	1	2	7	8	9	17	18	20
Clavicle	219 ± 16**	148 ± 15**	112 ± 11	103 ± 8	108 ± 9	114 ± 8	80 ± 6**	96 ± 6
Hind foot	208 ± 37**	198 ± 40*	75 ± 15	60 ± 13**	70 ± 13*	101 ± 36	71 ± 15*	50 ± 12**
Radius, ulna forefoot	170 ± 13**	173 ± 28*	88 ± 7	76 ± 3**	91 ± 8	98 ± 9	79 ± 9*	78 ± 4**
Skull	162 ± 9**	188 ± 10**	92 ± 9	69 ± 7**	84 ± 9	78 ± 6**	80 ± 5**	98 ± 3
Ribs	136 ± 7**	101 ± 11	85 ± 6*	73 ± 6**	91 ± 12	93 ± 10	83 ± 6**	101 ± 5
Scapula	128 ± 7**	111 ± 5*	101 ± 6	96 ± 5	98 ± 7	98 ± 6	105 ± 4	110 ± 3**
Sternum	101 ± 6	113 ± 14	134 ± 8**	109 ± 6	113 ± 15	111 ± 5*	99 ± 7	108 ± 3*
Vertebral column	93 ± 3*	100 ± 3	86 ± 3**	83 ± 3**	91 ± 5	101 ± 3	102 ± 3	101 ± [illegible]
Pelvis	81 ± 4**	83 ± 4**	115 ± 4**	134 ± 4**	115 ± 8*	104 ± 5	107 ± 4	107 ± 4
Tibia, fibula	69 ± 4**	73 ± 6**	98 ± 9	100 ± 7	98 ± 6	101 ± 6	102 ± 7	94 ± 3
Humerus	74 ± 4**	65 ± 7**	127 ± 4**	139 ± 6**	123 ± 11*	109 ± 6	113 ± 9	100 ± 2
Femur	63 ± 3**	64 ± 4**	115 ± 2**	126 ± 6**	109 ± 8	103 ± 5	108 ± 3*	98 ± 2

Statistical significance of differences against 100% level determined by t test * $p<0.05$, ** $p<0.01$

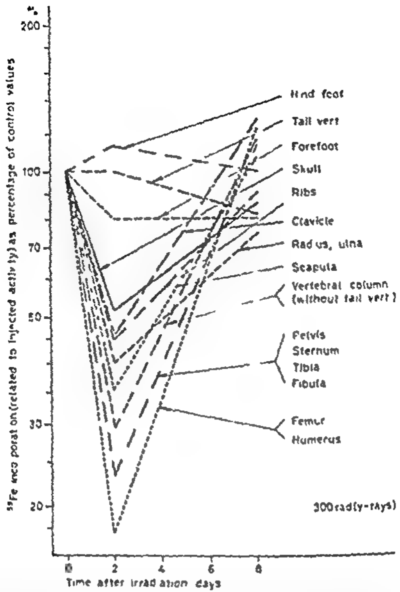


Fig. 2 Incorporation of ^{59}Fe (related to injected activity) into the marrow of individual skeletal parts at various time intervals after γ-irradiation with 300 rad exposure expressed as percentage of uptake in non irradiated mice

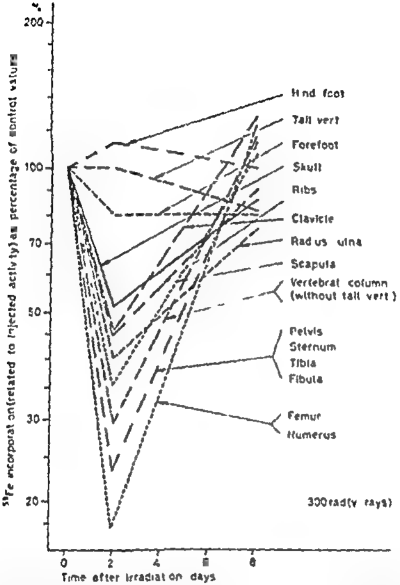
γ-irradiation in all skeletal parts is very close to that found after X-irradiation This correlation, both in the decreasing and the increasing phase, is of high significance (Spearman's coefficient of rank correlation r_s = 0 979 and 0 986, $p < 0.01$)

Results obtained after X irradiation with 100 and 500 r exposures will be described only summarily. After both exposures individual bones displayed different behaviour, as after irradiation with 300 r, the differences between bones in ^{59}Fe incorporation rising with increasing exposure. In order to decide whether the differences between individual bones are reproducible, a comparison was made of the rank order of decreases and that of increases of ^{59}Fe incorporation into the marrow of various skeletal regions at the time of maximal depression and maximal elevation, respectively, between individual experiments. The rank order of activity of individual bones during depression and recovery was similar after all three exposures. The rank order correlation [5] is highly significant during depression ($W = 0.905$, $F = 28.57$, $p<0.01$) and significant during recovery ($W = 0.457$, $F = 2.525$, $0.01<p<0.05$).

As apparently shown in figures 1 and 2 the rank order of the intensity of incorporation into the marrow of individual skeletal parts in the phase of post irradiational depression and in the phase of subsequent elevation is mutually dependent. In order to prove the validity of this hypothesis for all experimental data, the average rank values of incorporation into the marrow of individual skeletal parts were correlated, in the phase of post irradiational decrease on one hand and in the phase of post irradiation increase on the other, correlation is of high significance (Spearman's coefficient of rank correlation $r_s = -0.612$, $p<0.01$). Thus, it is evident that the skeletal parts reacting to acute irradiation with more pronounced decrease (femur, humerus, tibia and fibula, etc.) incline to a more pronounced elevation in the phase of the recovery of incorporation activity, on the other hand, a smaller increase is exhibited by skeletal parts reacting with a relatively lesser decrease after irradiation (skull, feet, etc.).

Discussion

Even though the intensity of ^{59}Fe incorporation into blood forming organs is currently used as an indicator of erythropoietic activity [6–8], for such an interpretation the presence of a fraction of varying magnitude of non haeme iron in erythropoietic tissue should be taken into consideration [9], as well as some further interfering ferro- and cytokinetic influences [10, 11]. As the ferrokinetic effects (for example, plasmatic Fe concentration, rate of Fe clearance from the plasma) are in our



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experimental arrangement the same for the bone marrow of all regions, and because the rate of Fe incorporation into non haeme Fe fraction in the undisturbed state of erythropoiesis does not exceed 10–20% of the total incorporation [12–14], our results obtained on *non irradiated* animals may be considered an approximate indicator of the participation of individual bones in the erythropoietic activity of bone marrow in the pre-irradiation stage (table I) Maximum incorporation activity of erythroid marrow occurs during the 3- to 6-hour period [1, 2] The proportion of the incorporation into the skeleton for a certain bone is constant at various intervals after isotope application Thus, the rate of ^{59}Fe incorporation during the 6 hour interval after application may be considered as a representative indicator of the total incorporation activity of the bone marrow of individual regions Our results, as shown in table I, are in close agreement with the data of other authors [1]

In contrast to the pre-irradiation stage, the relation between the rate of ^{59}Fe incorporation and intensity of erythropoiesis in individual skeletal parts *after irradiation* is somewhat equivocal According to our experimental findings [15] on C57BL/10 and BALB/c mice irradiated with 300 r X-ray exposure, the degree of post-irradiational decline and following overshoot of ^{59}Fe incorporation into the erythropoietic system resembles much more the decline and rise of the overall plasma iron turnover (PIT), than those of erythron turnover only (determined from PIT [16] It follows that the method of ^{59}Fe incorporation into erythropoietic organs is an indicator not only of the intensity of erythropoiesis, but also of at least part of the non-erythron turnover of Fe in those organs, in accord with other data [9] The differences, therefore, in post-irradiational behaviour of ^{59}Fe incorporation between the various regions of bone marrow, described in the present paper, may be conditioned not only by the different radiosensitivity of the erythropoietic tissue and its varying recovery ability, but also by the presence of fractions of various size of non-haeme (storage) pools of iron in individual bone marrow regions

From the experimental material presented it is clear that the marrow of the long bones, which is in haematology the most often used material, represents (at least from the point of view of radiosensitivity) the most 'reactive' part of bone marrow and is not, therefore, a representative indicator of overall marrow Fe incorporation In the period of post irradiational depression the Fe incorporation is maintained predominantly by the bones of spongiose type (skull, ribs, scapulae, etc) as follows

from this calculation, while in the resting state the long bones of the extremities and pelvis represent about 43% of the overall ^{59}Fe incorporation into the bone marrow, this value falls at the time of maximum postirradiational depression after 300 r X-ray exposure to 36% and after 500 r even to 26% The differences between the incorporation activities of bones of different types probably bear upon the particular microenvironment within them [17]

As irradiation of mice by means of ^{60}Co source gave at comparable exposure results identical to those obtained by means of X-rays, it may be stated that the differences observed in incorporation activity between various skeletal regions are not a consequence of special conditions of X-ray irradiation (distance of individual bones from radiation source, different thickness of the bone tissue, etc), but follow from different functional properties of the bone marrow in various regions of the skeleton itself

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Acta Haemat 52 161–168 (1974)

Congenital Dyserythropoietic Anemia with Ultrastructure Findings Compatible with both Types I and II

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Abstract A patient with congenital dyserythropoietic anemia with serological findings and electron microscopic features compatible with those of types I and II is described The anemia was characterized by erythroblastic multinuclearity, ineffective erythropoiesis, negative acidified serum test and negative sugar water test The cold antibody lysis test (anti I) was significantly positive and there was no evidence of anti i

Key Words
Dyserythropoietic anemia
Electron microscopy
Erythroblast ultrastructure
Erythropoiesis

The clinical hematological serological and electron microscopic studies of a patient with congenital dyserythropoietic anemia (CDA) type I, are described in detail Although the serological findings were compatible with those observed in CDA type I the el tron microscopic (EM) features were in accordance with those describ d in both types I and II The patient is followed up more than 2 years and did not show any changes in his clinical and hematological status

Methods

Routine hematological studies wer e f rmed according to Dacie and Lewis [4] Vitamin B_{12} in serum [6] folic a [7] in whole blood red cell enzymes [2] serology tests [17] erythro- and ferro] were performed as well

Aspirated bone marrow was imme placed in 1% glutaraldehyde in phosphate buffer pH 7.4, at 4 °C, postfixe m tetroxide dehydrate in graded alcohols and embedded in epon 812 (600 A) sections, obtained with an LKB ultratome III were examined with a Philips 300 electron microscope at 60 kV

Case History

B. E. is a 66-year-old male born in Morocco, married without children. He was admitted for the first time to another department in 1963 because of severe weakness. Physical examination revealed hepatosplenomegaly of 2 and 5 cm, respectively. His hemoglobin was 9.4 g/100 ml and 3.0% reticulocytes. In the blood film anisocytosis and poikilocytosis were observed. The bone marrow aspiration biopsy showed marked erythroid hyperplasia with numerous binucleated normoblasts. Treatment with repeated blood transfusions and occasional steroids did not improve his condition.

In 1973 he was admitted because of urinary tract infection and deterioration of his general condition in our department. The pertinent physical findings were splenomegaly and very mild jaundice. In the peripheral blood smear anisocytosis, poikilocytosis, polychromasia, basophilic stippling, occasional tear drop cells, spherocytes and normoblasts were observed. The bone marrow showed marked hyperplasia of the erythroid series. There were many binucleated normoblasts and some multinucleated normoblasts. Sideroblasts were demonstrable and iron stain showed increased amount of iron. No conspicuous changes were observed in white blood cells or platelets. Additional findings are presented in table I.

The serum iron was 144 μg/100 ml and TIBC 358 μg/100 ml. Serum bilirubin was 3.3 mg/100 ml (indirect 2.9 mg%). Haptoglobin level of the serum was low to zero. Hemoglobin electrophoresis showed no abnormalities. G-6-PD, pyruvate kinase and hexokinase activity of the red cells were normal. There was marked hyperuricemia (17.4 mg/100 ml) and the 24-hour fecal stercobilinogen excretion was 496 mg. Immunoelectrophoresis showed increase in IgG 2,300 mg/100 ml (normal 1,200 ± 300), IgA 700 mg/100 ml (normal 280 ± 100), IgM 150 mg/100 ml (normal 88 ± 22). Lactic dehydrogenase in the serum was 232 U/ml (normal 40–200).

Erythrokinetics. The ^{51}Cr T50 of the patient's red cells in his own circulation was 18 days (normal 25–33 days) and spleen/liver ratio was 1.9 (normal 1). Plasma

Table I. Hematological findings

Hb, g/100 ml	5.8–6.4
RBC × 10^6/μl	1.6
MCV, μm³	94–100
MCHC, %	38–40
Reticulocytes, %	0.4–3.8
Leukocytes/μl	8600
Platelets/μl	174 000
Osmotic fragility	*normal*
Osmotic fragility after incubation in 37 °C for 24 h	normal
Coombs direct, indirect	negative
Serum vitamin B_{12}, pg/ml	600 (normal 200–700)
Whole blood folic acid, ng/ml	62 (normal 100–300)

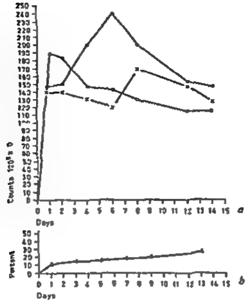

Fig. 1. Ferrokinetic studies with ^{59}Fe. *a* Surface counting over the marrow (sacrum) (o), spleen (●) and liver (×). *b* Red cell utilization expressed as percentage of the injected dose.

^{59}Fe clearance T50 was 20 min (normal 60–140 min). Plasma ^{59}Fe turnover 71 mg/24 h (normal 20–42 mg/24 h). ^{59}Fe incorporation after 7–10 days 21.8% (normal 80–90%). This data suggested ineffective erythropoiesis. The uptake of ^{59}Fe in the bone marrow (sacrum), spleen and liver and the iron utilization curve are shown in figure 1.

Serology. The patient's red cells were B, DCCee, I i[+ 1]. There was a negative acidified serum test with 3 sera while the cold antibody lysis (anti I)[2] was significantly positive ranging between 13 and 16% in a dilution of 1 in 100 [12]. The sucrose lysis test was negative and the acid lysis test with a panel of 5 donor sera of the patient's group (B) was repeated; at the same time parallel lysis test on red cells washed off clotted specimen was carried out. Both tests were negative. In addition there was no evidence of anti i.[2]

Electron microscopy. Abnormalities of nuclear division were found in polychromatophilic and orthochromatic normoblasts. A great number of them showed binu

[1] Kindly re-examined and confirmed by S. LEVINE, Central Laboratories, Ministry of Health, Jerusalem.

[2] Kindly performed by S. M. LEWIS, Postgraduate Medical School, Hammersmith Hospital, London.

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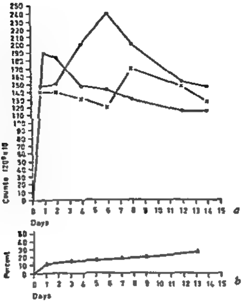

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Fig. 2. Electron micrograph of a binucleated normoblast. The cisterna parallel to the cell membrane is well demonstrated. ×13 100.

cleated nuclei (fig. 2) or incomplete division of the nuclei. Some of the nuclei showed a marked indentation. The chromatin was condensed, the nuclear membrane sometimes missing (fig. 2). The cytoplasm contained small round mitochondria, residual membranes (fig. 3) and well developed Golgi formation (fig. 4). Many cells showed circumferential cisternae which paralleled the plasma membrane (fig. 2, 3).

Discussion

The serological findings in this case correspond well with those of patients with CDA type I [8, 14]. According to previous reports, the nega-

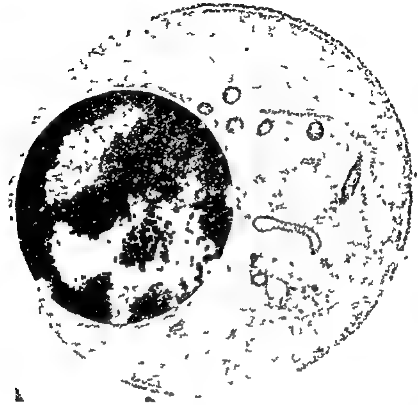

Fig 3 Electron micrograph of a normoblast with residual cytoplasmic membrane ×17 000

tive acidified serum test differentiates CDA type I from type II [3 13 14 19] If the anemia in our patient were CDA type II one would expect at least some normal sera to produce a positive acid lysis Our case could not be CDA type III [1 5 10] since in type of anemia there are gigantoblasts with erythroblastic multinuclearity

Ultrastructural findings in CDA type I are mainly consistent with nuclear defects such as wide pores in the nuclear envelope penetration of cytoplasmic material into the nucleus [8 11 14 15] It has been suggested that the hemoglobin which enters the nucleus through the wide nuclear pores interacts with nuclear chromatin and causes arrest of DNA syn

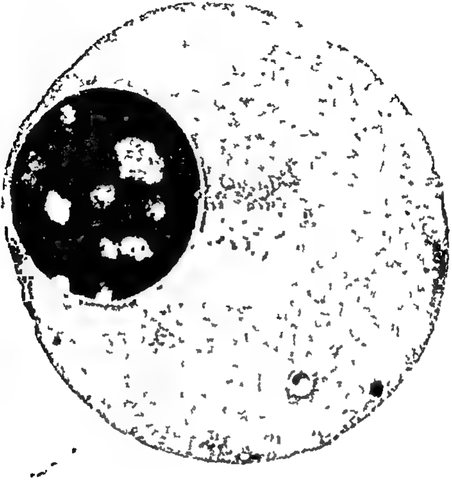

Fig. 4. An orthochromatic normoblast with very prominent Golgi formation. ×15 000

thesis in late normoblasts [15]. The ultrastructural appearance of the erythroblasts in our patient fitted well with these described in cases with CDA type I except for the finding of peripheral abnormal cysternae parallel to the cell membrane described in cases of CDA type II [9, 19, 20]. Since the serological tests in our case were compatible with those characteristic for CDA type I, the question is raised if this case is not an intermediate type between CDA type I and type II.

According to our knowledge, the present case represents an unusual type of CDA, i.e. with serological findings compatible with type I and electron microscopic features fitting with both types I and II.

Acknowledgement The skillful assistance of Mrs FISHMAN and Mr SADOVNIK is highly appreciated

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Acta Haemat. *52* 169–172 (1974)

Sideroblastic Anaemia Presenting as Monoarticular Arthritis

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Abstract A case of sideroblastic anaemia presenting as monoarticular arthritis is described whose affliction of the joint was due to excessive haemosiderin iron deposition in the synovial tissue The response of anaemia to pyridoxin therapy and improvement in arthritic involvement to administration of desferrioxamine has so far been remarkable

Key Words
Arthritis in sideroblastic anaemia
Haemosiderosis of synovia
Pyridoxin responsive anaemia
Sideroblastic anaemia

Anaemia characterised by the appearance, in large numbers, of abnormal sideroblasts in the bone marrow is classified as sideroblastic anaemia [8] Most patients present with progressive and resistent anaemia only, although haemosiderosis has also been described in some of them [1] Presentation with arthritis consequent to synovial siderosis has never been reported earlier The case described here is a contribution to the fact that with more experience and with better awareness of this condition, wider clinical spectrum is likely to emerge

Case History

M [illegible] a 38-year-old female had an 8-month history of pain and recurrent swelling of the left knee joint On admission she had an obviously swollen knee joint with painful and restricted movements No complaints or physical signs could be elicited in other joints There was no history of fever She was considerably anaemic Spleen liver or lymph nodes were not enlarged

Haemoglobin was 7 2 g% leucocytes 8 000/μl differential count P 60% L 33% M 4% and E 3% erythrocyte sedimentation rate 13 mm (1 h Westergren) packed


1


2

Fig 1 Extensive deposition of haemosiderin iron in the otherwise hypertrophied synovial membrane × 70

Fig 2 Iron deposits in synovial cells in the superficial areas × 250

cell volume 30%, mean corpuscular haemoglobin concentration 24%. Examination of the peripheral blood film showed dimorphic picture along with a large number of markedly hypochromic cells. Reticulocyte count 0.8%.

Bone marrow examination revealed macronormoblastic hyperplasia with the early cells showing megaloblastoid appearances. Most late normoblasts showed inadequately haemoglobinised cytoplasm and irregular cytoplasmic borders. Some showed prominent basophilic granules while others showed vacuolated cytoplasm. On iron staining, reticular iron was graded as + + +. More than 70% of the erythroid cells appeared as abnormal sideroblasts, of which 62% showed 'ringed' distribution of their siderotic granules.

Serum iron was 115 μg%, serum unsaturated iron binding capacity 230 μg%, plasma transferrin saturation 30.9% and serum bilirubin 0.8 mg%. Osmotic fragility was normal. Coomb's test was negative. Electrophoresis showed no abnormal haemoglobins. Alkali denaturation test and the test for G-6-PD deficiency were negative. Serum folate and serum vitamin B_{12} levels were normal.

Synovial fluid analysis was non-contributory in revealing the nature of arthritis and so was the X ray examination of the joint Latex fixation test was negative Synovial biopsy showed diffusely hypertrophied synovial membrane with scattered and mixed inflammatory reaction in the subsynovial and deeper tissue There were no distinct lymphoid aggregates or villous prolongations of the hypertrophied synovium Iron staining of the synovial biopsy specimen showed extensive and heavy depositoin of haemosiderin iron both in the hypertrophied synovium and in the deeper tissue (fig 1) The iron deposits were observed in the synovial cells in the superficial areas (fig 2) and the fibroblastic tissue in the deeper areas

The patient was treated with pyridoxin and folic acid to which initially there was little response but later, on larger doses of pyridoxin alone, an appreciable rise in the haemoglobin level occurred and has since been maintained between 12 and 13 g%/ for a period well over 1 year The improvement in general well being of the patient has been remarkable For arthritis, synoviectomy was performed followed by administration of the iron chelating agent Desferrioxamine (Ciba) at frequent intervals For the past year she has been free from arthritic symptoms

Discussion

According to the classification suggested by MOLLIN and HOFFBRAND [8], the type of sideroblastic anaemia presented by this patient is considered to be of 'primary acquired variety' The involvement of knee joint (monoarticular arthritis) seems to be the result of extensive synovial siderosis, consequent to the fundamental defect of the sideroblastic anaemia It is possible that iron deposition in the synovial tissue resulted in the liberation of lysosome from the synovial cells causing damage to the joint with consequent manifestation of arthritis Relief after synoviectomy combined with therapeutic administration of iron chelating agent is ample evidence of this hypothesis

The association of anaemia with rheumatoid arthritis is well known, wherein excessive deposition of haemosiderin iron in storage sites (bone marrow and liver) as well as in the synovial tissue has also been described [2] Rheumatoid arthritis has also been included amongst the causes of secondary sideroblastic anaemia [4] In the present case, however, the anaemia does not appear to be secondary to rheumatoid arthritis because of the following reasons (1) The arthritic lesion did not conform to the classical criteria of rheumatoid arthritis (2) Hypoferraemia and hypotransferrinaemia, which are characteristic of the anaemia of rheumatoid arthritis, were lacking (3) The appreciable response to pyridoxin therapy also points to 'primary sideroblastic anaemia'

The occurrence of haemosiderin iron in the synovial tissue has been observed in several other arthritic conditions too. These include tuberculous arthritis [7], traumatic arthritis [12] and haemophilia [3]. It is believed that synovial cells possess exceptional avidity for iron [6] and that synovial cells are capable of converting ferritin into haemosiderin *de novo*. The occurrence of synovial siderosis in primary sideroblastic anaemia as observed in the present case, should not appear surprising when it is realised that generalised haemosiderosis and even haemochromatosis have been observed in this condition [1, 2, 5]. However, this is the first instance that its presenting as monoarticular arthritis is reported.

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Acta Haemat 52 173-179 (1974)

Chromosomal Abnormalities in a Patient with Adolescent Myelofibrosis[1]

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Abstract All karyotypes of unstimulated blood leukocytes from a young adult patient with myelofibrosis-myeloid metaplasia contained 2 small extra metacentric chromosomes which may have originated in a leukocyte stem cell line by long arm breakage in chromosome 18 Aneuploidy of the C group F group and Y chromosome was also found in some unstimulated leukocytes Karyotypes of PHA stimulated blood lymphocytes and skin fibroblasts were normal

Key Words
Chromosome aberrations
Karyotype
Leukocyte chromosomes
Myelofibrosis
Myeloid metaplasia

Although chromosome studies of blood and marrow cells have been normal in the majority of patients with myelofibrosis myeloid metaplasia, abnormalities of C group chromosomes (numbers 6–11) have been demonstrated in peripheral blood leukocytes Extra C chromosomes [1, 2], as well as C group deletions [3, 4], have been associated in several patients with increasingly leukoblastic peripheral blood smears Additionally, reports of G group (numbers 21–22) abnormalities in myelofibrosis have recently appeared [5] Philadelphia chromosome (Ph^1) disomy in blood leukocytes was discovered concomitant with the detection of late onset

[1] Supported in part by NIH grant AM 05472-08 and by Project 903 Maternal and Child Health Services HEW Health Services and Mental Health Administration

[2] We appreciate the technical assistance of Miss GRACE ARIMURA, Mrs FLORA BOWENS and Mr REINALDO FERNANDEZ.

The occurrence of haemosiderin iron in the synovial tissue has been observed in several other arthritic conditions too. These include tuberculous arthritis [7], traumatic arthritis [12] and haemophilia [3]. It is believed that synovial cells possess exceptional avidity for iron [6] and that synovial cells are capable of converting ferritin into haemosiderin *de novo*. The occurrence of synovial siderosis in primary sideroblastic anaemia as observed in the present case should not appear surprising when it is realised that generalised haemosiderosis and even haemochromatosis have been observed in this condition [1, 2, 5]. However, this is the first instance that its presenting as monoarticular arthritis is reported.

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Abstract All karyotypes of unstimulated blood leukocytes from a young adult patient with myelofibrosis-myeloid metaplasia contained 2 small extra metacentric chromosomes which may have originated in a leukocyte stem cell line by long arm breakage in chromosome 18 Aneuploidy of the C group F group and Y chromosome was also found in some unstimulated leukocytes Karyotypes of PHA-stimulated blood lymphocytes and skin fibroblasts were normal

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Although chromosome studies of blood and marrow cells have been normal in the majority of patients with myelofibrosis-myeloid metaplasia, abnormalities of C group chromosomes (numbers 6–11) have been demonstrated in peripheral blood leukocytes Extra C chromosomes [1, 2], as well as C group deletions [3, 4], have been associated in several patients with increasingly leukoblastic peripheral blood smears Additionally, reports of G group (numbers 21–22) abnormalities in myelofibrosis have recently appeared [5] Philadelphia chromosome (Ph[1]) disomy in blood leukocytes was discovered concomitant with the detection of late onset

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myelofibrosis in 5 chronic myelocytic leukemia patients previously determined to have a single Ph[1] chromosome

We observed a case of adolescent myelofibrosis-myeloid metaplasia whose unstimulated blood leukocytes contained 2 extra metacentric chromosomes smaller than those of either the F or the G group. Aneuploidy in both the C and F group chromosomes was also observed.

Case Report

The patient, a 13 year old black male, presented in 1961 with fever, arm pain, generalized lymphadenopathy, and hepatosplenomegaly. Hemoglobin 11.7 g%, hematocrit 34%, white blood cells 124,000/μl with 17% segs, 24% bands, 5% lymphocytes, 32% metamyelocytes, 11% myelocytes, 6% promyelocytes, 5% myeloblasts and 2 nucleated red cells per 100 white blood cells. Platelet count 500,000/μl. Bone marrow was hypercellular. The most likely diagnosis was myeloproliferative disorder, perhaps chronic myelogenous leukemia. He was treated with cyclophosphamide, 6-mercaptopurine, azauridine, and streptonigran during the next several months.

He did well clinically during the ensuing 10 years, intermittently requiring short courses of busulfan to control leukocytosis and bone pain. Karyotype studies (discussed below) showed that the Ph[1] chromosome was not present in unstimulated blood leukocytes, and that blood T lymphocytes and skin fibroblasts were of normal karyotype. At age 23, the patient's hemoglobin and platelet counts began to decrease and bone marrow aspiration was unsuccessful. Liver and spleen were greatly enlarged. Hemoglobin 8.[illegible] g%, hematocrit 27%, white blood cells 24,000/μl with 15% segs, 25% bands, [illegible]% lymphocytes, 3% monocytes, 6% basophils, 15% myelocytes, 18% metamyelocytes, 10% myeloblasts and [illegible] nucleated red cells per 100 white blood cells. Platelets 110,000/μl. Peripheral smear showed giant platelets and tear drop-shaped red cells with marked aniso- and poikilocytosis and schistocytosis. Leukocyte alkaline phosphatase scores ranged from 60 to 185 (normal 10-90). Bone marrow biopsy revealed marked fibrosis and osteosclerosis.

In view of the patient's leukoerythroblastic anemia, normal leukocyte alkaline phosphatase scores, absent Ph[1] chromosome, fibrotic sclerotic marrow, and prolonged clinical course, a diagnosis of myelofibrosis-myeloid metaplasia was made. He was treated with androgens, however, he became progressively more anemic and thrombocytopenic. A splenectomy was performed with the removal of a 2,500 g spleen which histologically showed fibrosis and extramedullary hematopoiesis.

There were transient increases in hemoglobin and platelet counts following splenectomy, although the leukoerythroblastic anemia remained severe in spite of androgen-glucocorticoid therapy. Several months following splenectomy, a left subphrenic abscess developed and was drained at laparotomy. The patient died of sepsis on the first postoperative day in spite of intensive antibiotic therapy. Autopsy findings included extensive severe myelofibrosis and myelosclerosis, and a 4,900-gram liver with multiple hematopoietic foci.

Materials and Methods

Three cell types were cultured and chromosomes from each were analyzed on 3 occasions. Thirty cells were visually analyzed each time. Karyotypes were prepared using conventional techniques [6].

Chromosomal studies were done during the last year of the patient's life. He had, at that time, received cytotoxic drug therapy intermittently during the preceding 10 years. He had not, however, received radiation therapy or been examined with radionuclide diagnostic techniques when these studies were done.

Heparinized blood (5 ml) was diluted in 1000 ml colcemide (1 μg/ml) containing Hanks salt solution, incubated for 2 h at 37 °C, and unstimulated blood leukocytes were harvested [7]. Phytohemagglutinin (PHA)-stimulated thymus-directed blood lymphocytes [8, 9] were incubated and harvested by standard techniques [7]. Fibroblasts were cultured from skin and processed for chromosomes [10]. Subsequent to the death of the patient, slides previously prepared for karyotyping were destained in 95% ethanol and differentially stained by trypsin-Giemsa [11].

Results

Karyotypes of PHA-stimulated blood lymphocytes (fig. 1a) and skin fibroblasts (fig. 1b) were normal. However, all unstimulated blood leukocytes showed aneuploidy. Karyotypic data of these cells are presented in table I. All unstimulated leukocytes contained 2 extra metacentric chromosomes having arms approximately the length of the short arms of either chromosome 17 or 18 and morphology clearly distinguishable from Ph[1] chromosomes (fig. 1c–e). Giesma banding suggested that these extra chromosomes might be remnants of chromosome 18 with the terminal portion of the long arms deleted (fig. 1e, f).

We were unable to determine from the Giemsa-banded preparations which of the C chromosomes was in excess, or whether the 19 or 20 chromosome was missing in those unstimulated leukocytes showing aneuploidy. Both chromosomes 21 and 22 were normal in all karyotypes.

Table I. Aneuploidy of unstimulated blood leukocytes

Chromosome	Karyotypes, %
C^+	80
$meta^+meta^+$	100
Y^-	50
F	30

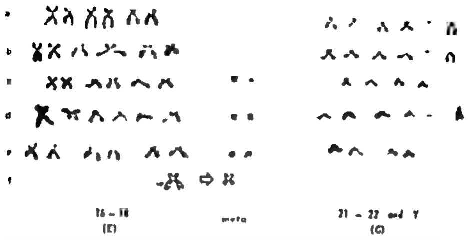

Fig. 1. a = PHA stimulated blood lymphocyte, b = skin fibroblast, c–e = unstimulated blood leukocytes. Leukocytes (c and e) X, C+, (d) XY. Karyotype (e) Giemsa banded. Proposed origin of extra metacentric chromosomes (meta) from chromosome 18 (f).

Discussion

A myeloproliferative process, first observed in this patient at age 13, terminated in profound leukoerythroblastic anemia 10 years later. Karyotype studies 10 years after the onset of clinical symptoms revealed the existence of 48 chromosomes in unstimulated peripheral blood leukocytes. The 2 extra chromosomes were metacentric and smaller than other chromosomes. To our knowledge no other patient with myelofibrosis-myeloid metaplasia has been found to have this type of chromosomal abnormality which is clearly distinct from the double Ph¹ chromosome patterns previously mentioned.

Our patient also had unstimulated blood leukocytes which were aneuploid for the C and F group and/or the Y chromosome. Other myelofibrosis patients have had aneuploidy in unstimulated blood leukocytes. Trisomy in the C group has been the chromosomal aberration most frequently found [1, 2, 12–15]. Disomy of the Ph¹ chromosome and C group deletions have been associated with increasingly leukoblastic peripheral blood findings during the course of myeloproliferative disorders [3, 5, 16, 17].

Extra metacentric chromosomes were present in all karyotypes from 3 cultures of unstimulated blood leukocytes during the last year of our pa-

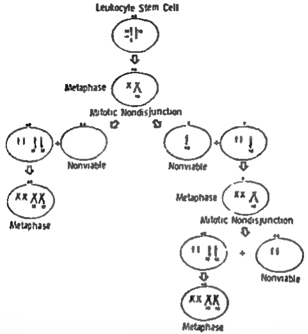

Fig 2 Breakage in one chromosome 18 followed by a single mitotic nondisjunction involving both normal and broken 18 (left), or 2 sequential mitotic nondisjunctions involving first the broken and then the normal 18 chromosome (right)

tient's life Giemsa banding revealed that the metacentric chromosomes were, most likely, fragments of chromosome 18 deleted of a portion of the long arm Breakage may have been followed either by one nondisjunctive mitotic division or by 2 sequential nondisjunctive divisions involving both normal and broken 18 chromosomes (fig 2)

An extra C group chromosome was detected in 80% of unstimulated leukocytes One mitosis with nondisjunction of a C chromosome must have occurred during leukocyte stem cell proliferation This abnormal mitosis was, apparently, subsequent to the nondisjunctional divisions which resulted in the appearance of the 2 metacentric chromosomes in the leukocyte line, since the leukocytes of V H were mosaic for C^+, but not for $meta^+meta^+$ Alternately, the single extra C might have been lost by anaphase lag from a proliferating leukocyte precursor after abnormal divi-

sions which resulted in the extra metacentric chromosomes The same argument holds for aneuploidy of the F and Y chromosomes

Myelofibrosis patients have been reported to have cells with various chromosomal alteration (e g , breakage, dicentrics, and chromatid alterations [12, 13], translocations [18], E group deletions [1], D trisomies [15]) in addition to C trisomy and Ph¹ disomy mentioned above Many of the reported patients with these abnormalities had received cytotoxic and/or radiation therapy

Our patient received chemotherapy intermittently during the 10 years preceding the initial detection of extra metacentric chromosomes in peripheral blood leukocytes However, morphological alterations were not found in the chromosomes from the 3 different cell types studied This observation, along with the normal karyotypes of PHA-stimulated blood lymphocytes and skin fibroblasts, speaks against drug induced chromosome injury as the explanation for the abnormal karyotypes in peripheral blood leukocytes [19, 20]

The finding of extra metacentric chromosomes in unstimulated peripheral blood leukocytes- but not in PHA-stimulated lymphocytes or skin fibroblasts – suggests an acquired, clonal chromosomal aberration in this patient Other workers have also demonstrated leukocyte karyotypic abnormalities in myelofibrosis patients HUMBERT *et al* [4] reported blood leukocyte C group deletion and karyotypically normal skin fibroblasts VAN SLYCK *et al* [18] found normal peripheral lymphocyte and marrow fibroblast karyotypes in a myelofibrosis patient whose unstimulated blood leukocyte culture included cells with a 1–3 translocation If these defects are related to chromosome injury by drugs, then the chromosomes of rapidly dividing leukocyte precursors must be more susceptible to alteration by these agents than are PHA-stimulated blood lymphocytes or skin fibroblasts

It is not known whether the chromosomal alterations demonstrated in unstimulated blood leukocytes from patients with myelofibrosis myeloid metaplasia are causally related to the disease process Patient V H is the first reported individual with E group aneuploidy in the unstimulated blood leukocyte line associated with karyotypically normal blood lymphocytes and skin fibroblasts

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Acta Haemat. 52: 180–188 (1974)

Haemoglobin G St. José in an Italian Family

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Abstract. Hb G St. José – β_7 (A4) glutamic acid → glycine – was observed in a Sicilian mother and daughter. The former was clinically and haematologically normal, whereas the daughter manifested severe haemolytic anaemia. A relationship between this and Hb G was excluded on chemical and physical grounds.

Key Words
Hb G St. José
Haemoglobinopathies
Haemolytic anaemia

Hb G was first described by Edington and Lehmann [1] in an African with an abnormal haemoglobin whose migration pattern was similar to that of Hb I. A similar electrophoretic picture was then noted by Schwartz *et al.* [2] in a subject of Italian origin (Calabria) resident in California. This patient was a double heterozygote for Hb G and Hb S and his erythrocyte morphology manifested several typical β thalassaemia features. It was not until 1960 that the amino acid substitution – β_7 (A4) Glu→Gly – was identified by Hill *et al.* [3] and the name Hb G St. José assigned. At alkaline pH, migration is intermediate between Hb A and Hb S and very similar to that of Hb F. In the only case so far reported, the abnormal haemoglobin formed 40% of the total haemolysate. Hb G variants with different substitutions have since been described. A complete list has been published by Lehmann [4]. A case of double heterozygosis between Hb G and β thalassaemia has also been reported in Italy [5]. Here, however, the exact amino acid substitution was not described.

Case Report

The proposita was a 2 year-old girl of Sicilian origin (Catania) with severe haemolytic anaemia spleen and liver enlargement increased blood bilirubin marrow erythroid hyperplasia with signs of megaloblastosis reticulocytosis aniso- and poikilocytosis, polychromasia, stippled target and fragmented cells and increased red cell osmotic resistance The general picture also included dystrophy and malnutrition following prolonged serious neonatal icterus The mother presented the same abnormal haemoglobin but her haematological and clinical picture was normal The data for both mother and daughter are collected in tables I and II The father was apparently healthy but refused to cooperate

Methods

Standard blood tests were run and osmotic resistance was determined with Simmel s method Cellulose acetate electrophoresis was carried out with glycine buffer pH 8 6 After elution with distilled water the fractions were read spectrophotometrically at 415 nm [6]

The abnormal haemoglobin was separated by chromatography on a 2.5×100 cm Sephadex DEAE A 50 column using the technique of HUISMAN and DOZY [7] Following vacuum concentration the α and β chains were separated by incubation with p-chloromercuribenzoate (PCMB) [8] and examined electrophoretically on cellulose acetate Finger prints were taken on total globin and on the aminoethylated (A/E) α and β-chains [9 11] The abnormal peptide (βI) was eluted with HCl 6 N and hydrolyzed at 110 C for 18 h followed by examination in an Optica analyzer Its sequence was studied by elution with NH_4HCO_3 0.5 M and digestion of the eluate at 38 C with a carboxypeptidase B solution using an enzyme substrate ratio of 1 50 [12] Aliquots were collected in 5 30 and 60 min, and the reaction was stopped with acetic acid Released amino acids were identified by high voltage electrophoresis at pH 2 and their amounts estimated on the Optica analyzer

Sickling was determined with 2% sodium metabisulphite [13] Solubility alkali resistance heat lability and the formation of methaemoglobin were evaluated by the methods of ITANO [14] BETKE et al [15] DACIE et al [16] and EVELYN and MALLOY [17] respectively Absorption spectra between 450 and 650 nm were carried out with $^{1}/_{15}$ M phosphate buffer pH 6 8 as described by DUBOWSKI [18] Prolonged exposure of the haemolysate to atmospheric air was used to obtain oxygenation while deoxyhaemoglobin methaemoglobin and cyanmethaemoglobin were obtained by adding sodium metabisulphite ferricyanide cyanide and potassium ferricyan de respectively

Globin chain synthesis rates were determined by incubating peripheral blood with ^{14}C leucine for 120 min and evaluating the uptake in each chain [19] Erythroid cell kinetics were determined on the bone marrow blood by incubation with 2.5 μCi ^{3}H thymid ne for 60 sec followed by fixation and autoradiography

The percentage of labelled cells was determined on 1500 erythroblasts Red

Table I

	Hb electrophoresis, %				Hb g%	RBC $\times 10^6/\mu l$	Ht %	MCV μm^3	MCHC %	Retics. %	Red cell morphology			Spleen and liver enlargement
	A_2	Γ	A	G							A	P	T	
Proposita	2.0		63.0	35.0	3.1	1.4	10	71.4	32	8.0	+	+	+	+ +
Mother	2.6		60.4	38.0	12.4	4.2	41	95.0	32	1.3	–	–	–	absent

A = anisocytosis, P = poikilocytosis, T = target cells

Table II

	Simmels test 0 35 % NaCl	Sickling	Plasma bilirubin mg %	Plasma iron µg %	Coombs' test	Associated diseases	Diagnosis
Proposita	+	neg	2 2 con 0 7 uncon	100	neg	nutritional dystrophy	A/G heterozygosis associated with nutritional dystrophy
Mother	neg	neg	–	–	neg	–	A/G heterozygous carrier

Table III Amino acid analysis of the abnormal peptide (30 6 nmoles/residue)

Amino acid residue	Normal β-TpI	Abnormal β TpI
Threonine	1	1 04
Glutamic acid	2	1 12
Proline	1	1 15
Glycine	–	0 93
Valine	1	0 91
Leucine	1	1 11
Lysine	1	1 07
Histidine	1	1 04

cell glycolytic enzyme activity was examined in accordance with the procedure proposed by Pescarmona *et al* [20]

Results

Haemoglobin electrophoresis of the proposita is shown in figure 1 Hb G amonts to 35 of the total, A_2 is within the limits of normal, and F is overlaid by the pathological fraction and hence invisible

PCMB *investigation showed normal* α *and slow moving* β *chains* (fig 2) The finger-print of the purified globin G was marked by replacement of the normal positive histidine βI by a new positive histidine peptide between αX–XI and αI–II (fig 3) As expected the finger-print for the isolated and aminoaethylated chains confirmed this picture

Fig. 1. Cellulose acetate electrophoresis (pH 8.6). From left to right: two normal subjects, the proposita, and a double heterozygosis HbC-β-thalassaemia

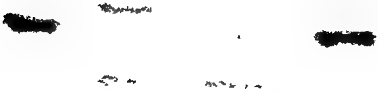

Fig. 2. Cellulose acetate electrophoresis (pH 8.6). From left to right: Hb A, Hb A after incubation with PCMB, Hb G St. José after incubation with PCMB, Hb G St. José. Note decreased mobility of Hb G-β-chains

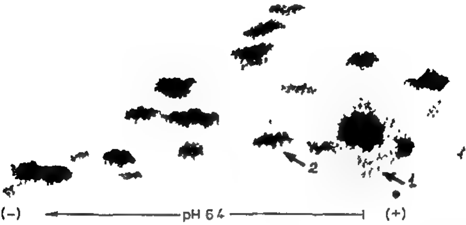

Fig 3 Finger print (pH 6.4) of Hb St Jose 1 = Absence of normal βI 2 = βI in Hb G St José

Amino acid analysis showed that the abnormal peptide had only one glutamic acid instead of the two observed in Hb A peptide (table III). Glutamic acid residues are observed in both position 6 and position 7 of βI. Further analysis showed that its sequence included a lysine as C-terminal amino acid and a penultimate glycine. The quantitative data for this sequence are shown in figure 4.

Functional data for the Hb G St Jose oxy-, deoxy-, met-, and cyanmethaemoglobin absorption spectra were normal. Methaemoglobin level was 25–43% in the purified Hb G, while it was 4–14% in the purified Hb A. In addition, the purified Hb G displayed a small degree of heat lability, with 36–40% of precipitated Hb, while it was 10–12% in the purified Hb A.

Synthesis of the globin chains showed that the abnormal β-chains were 46% of the totale β-chains, while the α/β-ratio was 1.08 (fig. 5).

Labelling index of red cells: proerythroblasts 73.77%, basophilic erythroblasts 72.01%, polychromatic erythroblasts 26.15%, orthochromatic erythroblasts 0%. The proposita's glycolytic enzyme and red cell substrate data were: pyruvate kinase 211 μM/h/ml red cells/25 °C, glucose-6

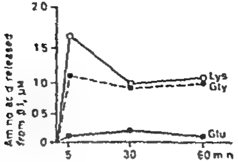

Fig 4 Rate of carboxypeptidase induced release of amino acid from β1 of Hb G St José

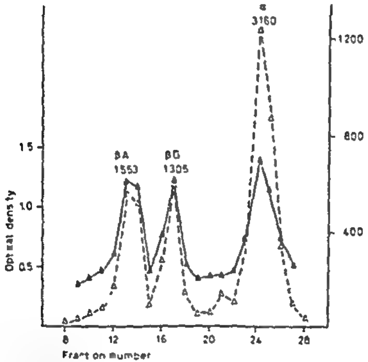

Fig 5 Elution pattern of radioactivity (—) and optical density (▲-▲-▲) in globin chains obtained by CMC chromatography (proposita) The numbers by the peaks indicate the total amount of radioactivity taken up by each single chain

phosphate dehydrogenase 200 μM h ml red cells 25 C, hexose kinase 25 μM/h ml red cells 25 C, ATP 1,180 nM ml red cells, glutathione 2,050 nM ml red cells, 2,3-diphosglycerate 4 600 nM ml red cells

Discussion

The amino acid mutation – β_7 (A4) glu→gly – observed in these two cases [illegible] that described for Hb G St José. Since it causes [illegible] loss of negative charge in βI, the abnormal molecule migrates more slowly than Hb A [illegible] pH 8 [illegible] and βI increases its cathodic mobility in the finger print. In the family observed by SCHWARTZ *et al.* [2] Hb G St José was functionally normal. In our proposita, however, slight heat [illegible] and increased methaemoglobin in the purified fraction were noted. This may be due to the formation of methaemoglobin A during chromatography and its contamination of Hb G, since it has been shown to display great[illegible] instability than Hb A and migrate in the same way as the abnormal haemoglobin [21–23]. α/β-chain ratio was 1.09 and [illegible] G chain synthesis was [illegible] of the total β-chains, i.e. little different from the relationship between these two fractions. Erythroid cell kinetics was at the upper limits of normal.

[illegible] can be seen, therefore, that Hb G St. José is not itself a cause of anaemia, since its physical and chemical characteristics are similar to those of the normal adult haemoglobin. The association of [illegible] in our proposita may be looked upon as a coincidence, particularly since the mother had the same abnormality and normal blood chemistry.

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Acta Haemat. 52 189–192 (1974)

Hexagonal Arrangement of Intragranular Particles in Human Basophilic Leucocytes

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Abstract The electron microscopic study of leucocytes from a child with a familial cerebroretinal degeneration disclosed a hexagonal array of the intragranular particles of some basophiles This highly ordered arrangement, which seems not to have been rep ed before most probably represents an incidental find bearing no relation either to the patient s basic cond t r to the anticonvulsive treatment

Key Words
Basophilic leucocytes
Electron microscopy
Ultrastructure of basophile granules

The literature on the ultrastructure of human basophiles comprises less than 20 titles The m ture granules of the glutaraldehyde-osmium fixed human basophile w apart from an occasional membrane whorl and a patch of moderat e amorphous material, a particulate content with or without one or ncentric rows of rounded particles lined up along the limiting mem the granule [1–4, 8] The filamentous formation with a 40 Å perio d in the central region of the granules has remained an isolated obse In this paper we report on a highly ordered arrangement of th nular particles in the basophiles of a child with an unidentified form retinal degeneration

Materials and Methods

Case report The p year-old girl with arrested psychomotor development since the age of m ic seizure activity and optic atrophy Routine blood smears and blood cell elded normal results A younger sibling of the patient suffers from the same l boratory tests biopsy tissue studies and an electron microscopic study of peripheral blood were all noncontributory from a

diagnostic viewpoint. The patient has been receiving supportive therapy including anticonvulsive medication.

Electron microscopy of peripheral blood. 10 ml of heparinized blood was drawn from the patient's left jugular vein with a plastic syringe. The blood was immediately transferred to a 100 × 13 mm test tube and spun down at 2,500 rpm for about 25 min at room temperature. The plasma was decanted and the tube was filled with 2.5% phosphate buffered glutaraldehyde solution and stored overnight at 4°C. The buffy coat was diced into small blocks and these were postfixed with 1% osmium tetroxide for 3 h. Blocks were dehydrated in graded ethanol solutions and embedded in Epon 812. Sections were cut with a diamond knife on an LKB Ultrotome, placed on uncoated copper grids, double-stained with uranyl acetate and lead citrate and examined with a Zeiss EM 9A electron microscope at initial magnifications ranging from ×1,900 to ×41,000.

Observations

Basophilic leucocytes displayed all of the usual electron microscopic features including those concerning number, shape, size and internal organization of granules (fig. 1, 2). The only unusual feature was the occurrence of a rare granule with a highly ordered particulate content variably patterned depending upon the orientation of the section with respect to the planes of the intragranular lattice (fig. 2). Essentially, the particles formed either a hexagonal array of discrete, dense, globular units about 170 Å in diameter, with a center-to-center spacing of approximately 240 Å, or, a banding pattern of straight, parallel, about 170 Å wide, beaded linear densities separated by light, narrow bands intervening between them. Upon examining several electron micrographs one got the impression that the continuous dense lines resulted from off register superimposition of rows of closely spaced globular units (fig. 2, inset). Finally, coextension of granule profile and lattice and banded patterns was seen only once; restriction of such patterns to a variably circumscribed area of a few granules was otherwise the rule.

Discussion

This seems to be the first report of a crystalline arrangement of the intragranular particles in human basophiles. Several types of periodic structures have been described in the basophilic granules of other species [5, 6], however, none of them seems to match exactly the finding herein reported. It is

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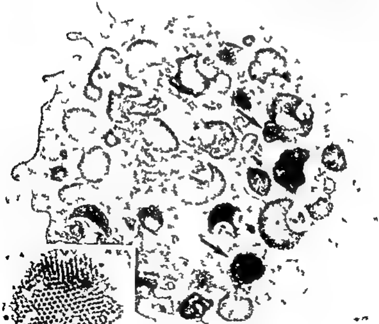
Fig. 1. Survey electron micrograph of peripheral blood buffy coat leucocytes. A basophile is seen in the left lower corner. ×4,750.

Fig. 2. Basophile from the peripheral blood. The plane of section passes off the central nuclear area of the cell. Granules displaying a highly ordered particulate arrangement are arrowed. ×18,000. *Inset.* High magnification of one of the arrowed granules: the lattice and banded patterns of the particles are evident. ×54,000.

1



2

very unlikely that this regular array of the intragranular particles in human basophiles may have anything to do with either the patient's basic disease or with the anticonvulsive therapy. It appears rather likely that we are dealing with an incidental finding in some isolated granules, possibly depending on some local factors.

Acknowledgements. We thank Mrs. P. Becker, Mrs. A. Heinzinger and Mrs. U. Orini for their collaboration.

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Acta Haemat 52 193–200 (1974)

Extrarenal Lipid Inhibitors of Human Erythropoietin

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Key Words
Erythropoiesis
Erythropoietin inactivation
Lipid inhibitors of erythropoietin
Stomach lipids

Abstract The assumption that inability to extract erythropoietin from the kidney is due to a renal lipid inhibitor, which inactivates the hormone during the process of extraction, has been substantiated Discovery of erythropoietic inhibitory activity in the spleen and liver prompted this comparative study of other extrarenal tissues for erythropoietic inhibitory factor but also as a means of examining organ specificity for binding the hormone Rabbit stomach lipid extracts proved to be even more active against erythropoietin than kidney extract Other tissues exhibited moderations of erythropoietic inhibitory activity, though brain and subcutaneous fat were decidedly inactive against erythropoiesis However, this hypothesis must rest on reproducible *in vivo* assessment of the inhibitor

During the past 60 years sufficient evidence has accumulated to substantiate the existence of erythropoietin and to factualize its essential role in normoblastic erythropoiesis Parri passu with advancing development of information about erythropoietin had been the necessity for its extraction from the most probable site of production, the kidney, in the main, this effort has hitherto been futile The inability of workers in the field of experimental haematology to successfully extract the hormone from the kidney has been attributed to several causes, the most significant of which must be those proposed by KURATOWSKA [8], FISHER *et al* [6], and ERSLEV and KAZAL [3] that the kidney produces an erythropoietic inhibitor which is capable of neutralizing the activity of erythropoietin during the process of extraction KAZAL *et al* [7] have demonstrated the lipid nature of the renal inhibitor It is a lipid which is active at both 37 and 4 °C The analysis of ERSLEV [4] has shown that 150 g of rabbit kidney yields about

very unlikely that this regular array of the intragranular particles in human basophiles may have anything to do with either the patient's basic disease or with the anticonvulsive therapy. It appears rather likely that we are dealing with an incidental finding in some isolated granules, possibly depending on some local factors.

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Acta Haemat 52 193-200 (1974)

Extrarenal Lip ˈubitors of Human Erythropoietin

Ko ES RAPHAEL ABAIDOO

Cardeza Foundation f ic Research Thomas Jefferson University Philadelphia, Pa

Abstract The assumpti to extract erythropoietin from ie to a renal lipid inhibitor w e hormone during the process tra t been substantiated Discovery rith inhibitory activity in the n and prompted this comparative st f other al tissues for erythropoietic inhi bitory factor but also as a me examin n specificity for binding the hor mone Rabbit stomach lipid ex proved en more active against erythro-poietin than kidney extract tissues exh moderations of erythropoietic inhibitory activity, though br nd subcu fat were decidedly inactive against erythropoiesis However hypothesis est on reproducible *in vivo* assessment of the inhibitor

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5 g of lipid erythropoietin inactivator, a finding which suggests that approximately 10,000 units of erythropoietin could be present in an inactive form in the renal homogenate from one kidney The quest of eventual separation of erythropoietin from the inhibitor is a pertinent one, since it could greatly ameliorate the anemia of chronic renal disease which presently is handled with androgens In view of the similarity of action of the inhibitors in other extrarenal tissues as described by ABAIDOO [1] and by ABAIDOO *et al* [2] it was deemed of interest to test ether solubility of the inhibitor principle as described in other tissues

Materials and Methods

Human urinary erythropoietin was supplied through the courtesy of the International Committee on Erythropoietin as procured by the Department of Physiology University of the Northeast Corrientes Argentina and processed by the Haematology Research Laboratories of Los Angeles Children s Hospital Distribution was authorized by the National Heart and Lung Institute under Research Grant HL 10880

Tissue Extracts Homogenates were prepared from stomachs intestines lungs kidneys spleens livers skeletal muscles hearts subcutaneous fat and brains of normal white New Zealand male rabbits weighing approximately 3 kg The tissues were minced suspended in icecold 0 02 mol NaCl (1 ml/g tissue) and homogenized in a Waring Blendor using 0 1 N NaOH or 0 1 N CHl the pH of the homogenate was adjusted to 7 2 and sonicated 3-5 sec with Heats Systems Sonifier, using bursts of about 30 W output The procedures were carried out at 4 °C with intermittent chillings in an ice bath The homogenate was screened through a fine stainless steel mesh or gauze and ultracentrifuged in the Beckman Model L with rotor No 40 at 40 000 rpm for 2 h (140 000 g) The supernatant was poured off and frozen at 20 C The precipitate was then made up to the original volume with 0 02 M NaCl and frozen in dry ice bath in acetone Subsequently the frozen material was lyophilized The lyophilized material was then rendered powdery with mortar and pestle and the ensuing powder was extracted with 50 ml saturated redistilled ether (2.5 ml g) for 30 min The extraction was repeated four times so that a total of 200 ml ether was used The supernatant was first filtered through a Whatman No 2 paper using a Buchner funnel with vacuum and then through Whatman No 12 in Erlenmeyer without vacuum The ether was then evaporated via water aspiration The lipid content of each tissue was then determined a record of which is depicted in table I

Experimental Procedure Hypoxic polycythemic ICR (International Cancer Research) female mice were used in this study The mice were pretreated for polythemia as follows prior to introduction into the hypoxic chamber each mouse received 1 mg iron dextran (Imferon®) intramuscularly Hypobaric hypoxia induced polycythemia was achieved by exposure of the mice to a simulated altitude of about 18 000 ft (0 4 atmosphere 304 mm Hg) in a steel-enclosed cylindrical decompression chamber whose capacity was approximately 18 ft³ in a room maintained at about 76 F The chamber is equipped with a Welch Duo-Seal Vacuum Pump

Table 1 Erythropoietic inhibitor activity of ether soluble fractions of rabbit tissues

Tissue	Amount injected with 1 U erythropoietin, equivalent of fresh tissue g	Amount of lipid, mg	^{59}Fe utilization, %, mean ± SD	Number of mice per assay
Saline	–	–	11 69 ± 2.52	15
Stomach	2.0	17 50	0 50 ± 0.23	6
	1 0	8 75	[illegible] 50 ± 0 16	[illegible]
	0 2	1 75	9 66 ± 1 44	6
Intestine	2.0	13 00	1 33 ± 0 48	8
	1 0	6 50	1.21 ± 0 26	[illegible]
	0.2	1 30	3 18 ± 0 90	6
Kidney	2 0	39 80	1 13 ± 0 54	7
	1 0	19 40	2.31 ± 0 83	7
	0 2	3 88	5 61 ± 4 60	8
Liver	2 0	25 00	1 18 ± 0 45	8
	1 0	12 50	3 45 ± 1.26	8
	0 2	2.50	9 94 ± 1 65	7
Spleen	2 0	100 00	2.10 ± 0 35	8
	1 0	50 00	3 24 ± 0 39	8
	0 2	10 00	8 93 ± 0 69	8
Skeletal muscle	2.0	2.50	8 15 ± 2 37	8
	1 0	1 25	7 90 ± 1 47	7
	0 2	0 25	11 67 ± 1 56	8
Lung	2 0	10 00	9 60 ± 1 20	[illegible]
	1 0	5 00	6 94 ± 1 18	6
	0 2	1 00	15 12 ± 2.15	7
Heart	2.0	150 00	9 06 ± 1 36	8
	1 0	75 00	11.26 ± 1 [illegible]	8
	0 2	15 00	12 39 ± 2.28	8
Brain	2 0	105 00	11 10 ± 3 61	8
	1 0	52 50	13 20 ± 1 87	8
	0 2	10 50	14 38 ± 0 93	7
Subcutaneous fat	2 0	98 00	13 39 ± 2.39	7
	1 0	49 00	10 11 ± 2.42	7
	0 2	9 80	10 03 ± 6 64	7

5 g of lipid erythropoietin inactivator, a finding, which suggests that approximately 10,000 units of erythropoietin could be present in an inactive form in the renal homogenate from one kidney. The quest of eventual separation of erythropoietin from the inhibitor is a pertinent one, since it could greatly ameliorate the anemia of chronic renal disease which presently is handled with androgens. In view of the similarity of action of the inhibitors in other extrarenal tissues as described by Abaidoo [1] and by Abaidoo *et al.* [2], it was deemed of interest to test ether solubility of the inhibitor principle as described in other tissues.

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Tissue Extracts. Homogenates were prepared from stomachs, intestines, lungs, kidneys, spleens, livers, skeletal muscles, hearts, subcutaneous fat, and brains of normal white New Zealand male rabbits weighing approximately 3 kg. The tissues were minced, suspended in icecold 0.02 mol NaCl (1 ml/g tissue), and homogenized in a Waring Blendor, using 0.1 N NaOH or 0.1 N CHl, the pH of the homogenate was adjusted to 7.2 and sonicated 3.5 sec with Heats Systems Sonifier, using bursts of about 30 W output. The procedures were carried out at 4 °C with intermittent chillings in an ice bath. The homogenate was screened through a fine stainless steel mesh or gauze and ultracentrifuged in the Beckman Model L with rotor No. 40 at 40,000 rpm for 2 h (140,000 *g*). The supernatant was poured off and frozen at −20 °C. The precipitate was then made up to the original volume with 0.02 M NaCl and frozen in dry ice bath in acetone. Subsequently the frozen material was lyophilized. The lyophilized material was then rendered powdery with mortar and pestle and the ensuing powder was extracted with 50 ml saturated redistilled ether (2.5 ml/g) for 30 min. The extraction was repeated four times so that a total of 200 ml ether was used. *The supernatant was first filtered through* a Whatman No. 2 paper using a Buchner funnel with vacuum and then through Whatman No. 12 in Erlenmeyer without vacuum. The ether was then evaporated via water aspiration. The lipid content of each tissue was then determined, a record of which is depicted in table I.

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Table II Erythropoietin inhibitor activity of ether soluble fraction from 2 g of normal rabbit tissues

Tissue	^{59}Fe utilization %[1] mean ± SD	Inhibitor units
Controls		
1 U erythropoietin	11.69±2.52 (15)	
Saline	0.40±0.02 (35)	
Stomach	0.50±0.23 (6)	0.98
Kidney (whole)	1.13±0.54 (7)	0.93
Liver	1.18±0.45 (8)	0.93
Intestine	1.33±0.48 (8)	0.91
Spleen	2.10±0.35 (8)	0.87
Skeletal muscle	8.15±2.37 (8)	0.48
Heart	9.06±1.36 (8)	0.37
Lung	9.60±1.20 (6)	0.33
Brain	11.10±3.61 (8)	0.00
Subcutaneous fat	*13.39±2.39 (7)*	*0.00*

[1] Numbers in parenthesis designate assays performed

Table III Analysis of variance of ether extracts of tissues at the 2 gram-equivalent concentration

Source of variation	Sum of squares	Degree of freedom	Mean square	F	p(F)
Among groups	[illegible] 35398	9	2.03933	101.44	<0.001
Within groups	1.28671	64	0.02010		
Total	19.64069	73			

s = 0.02010 = 0.142 antilogarithms = 1.39 coefficient of variation = 39%

The studies show that ether soluble fractions of brain and subcutaneous fat are completely devoid of the inhibitory principle. It shows that in the hypoxic assay model, stomach lipid is decidedly more potent as an erythropoietin inhibitor than the kidney, as shown in table II. The study confirms the assumption that the inhibitory principle is ether soluble in

(Model 1405, Sargent-Welch Scientific Company) with an intake valve which opens to normal atmospheric pressure and allows a flow through of some 1.7 ft^3 air/min at 0.4 atmospheric pressure. On day 11 the mice were subjected to 0.4 atmosphere 19 h/day and all weekend for 14 days. The chamber was opened daily for cleaning and refurbishing of feed and water. Each tissue was assayed in three groups of at least five mice/group. On days 5 and 6 post hypoxia, each group recieved, subcutaneously, 0.5 ml of 1 U human urinary erythropoietin incubated at 37 °C for 30 min with the ether equivalent of 2 g of the respective tissue (the lipid content (mg) is shown in table I). On day 7, each group received 0.5 μCi ^{59}Fe intraperitoneally in 0.2 ml of 0.9% isotonic sterile saline and on day 10, the 66-hour ^{59}Fe utilization was determined. All mice showing an hematocrit of less than 55% were discarded from the study.

Results

The assay showed complete neutralization of 1 U human urinary erythropoietin by stomach ether soluble fraction at the 2-gram equivalent level. 98% inhibition of erythropoietin was achieved with 2 g and 1 g equivalents of stomach lipid. However, at the 0.2-gram equivalent level, a ^{59}Fe utilization of 9.66% was recorded. This value represented merely some 33% inhibition of the activity of 1 U erythropoietin, which contrasts drastically with the recorded activity of the same concentration of stomach homogenate capable of neutralizing 94% of the erythropoietin's activity [1]. The 2-gram equivalent of ether soluble fraction of the whole kidney showed the potency of 93% inhibition of erythropoietin with a recorded radio-iron incorporation of 1.13%. This was slightly less active when compared with the inhibitory power of 2 gram of whole kidney homogenate [1] with a recorded radio-iron incorporation of 0.93%, though this difference is not significant. The activity of a 2-gram equivalent of liver tissue ether soluble fraction was very close to that of whole kidney, since both showed a 93 percent inhibition of human urinary erythropoietin. Not much difference could be seen between the inhibitory action of liver homogenate and the ether soluble fraction at the 2-gram level, nor was there much difference between them at the 0.2-gram level, both levels showing approximately 32% inhibition of the hormone. Ether soluble fractions of intestine, kidney, liver, and spleen, though not as potent as stomach fractions, showed moderations of inhibitory activity as depicted in table I. Sparse inhibitory activity was recorded from lung, heart, and skeletal muscle fractions, while brain and subcutaneous fat extracts were decidedly inactive against erythropoietin at all three levels of concentration tested.

at least primarily, to be associated with the kidney. However, since ERSLEV and KAZAL's [3] report of moderate erythropoietic inhibitory activity in spleen and liver homogenates, it has become a pertinent quest to survey other extrarenal tissues for possible presence of inhibitory principle. The nine tissues studied here (apart from the kidney) were therefore chosen randomly, since they could not be directly linked with erythropoietin production. In an earlier study, the objective had been to offer a comparative study of rabbit tissues with regard to erythropoietin inhibition; this study was to confirm ether solubility of the inhibitory principle. But secondly, it was evident that if erythropoietin were bound to a lipid inhibitor, as had been found in the case of the kidney, then a study of these extrarenal tissues could offer a means of learning about organ specificity for binding the hormone, and perhaps eventually of attempting to relate such observations to the general control of production and release of erythropoietin.

It may be conceded that this sort of assay is probably not adequately sensitive to discern a proper order of inhibitory activity within the group presented in this study, but the assay shows that, whereas brain and subcutaneous fat are completely devoid of the inhibitory principle, the other tissues showed moderations of activity.

Extrarenal erythropoietic inhibitory activity found in these studies suggest that one would have to interpret an erythropoietic stimulus in terms of a balance between stimulation and inhibition of the hormone with regard not only to the kidney. Consequently, overproduction of erythrocytes in polycythemia could be due to a defective inhibitory mechanism, an assumption which gives the inhibitor an essential homeostatic role in red cell physiology. Yet, the role of the inhibitor in endogenous regulation of red cell production and release is still a matter of controversy. This is because hitherto the activity of the lipid inhibitor has only been ascertained in *in vitro* studies; a physiologic homeostatic function must be viewed in terms of *in vivo* systems and as yet the inhibitor does not lend itself to *in vivo* assessment.

Acknowledgements

The invaluable technical assistance of Mr. ORIN P. MILLER of the Cardeza Foundation for Hematologic Research is appreciated. This study was supported by NIH grants No. 4612 and 6374.

IV Significance of differences between geometric means of values for organs in the 2 gram ether e[x]tracts

s		*Stomach*	*Whole kidney*	*Liver*	*Intestine*	*Spleen*	*Skeletal muscle*	*Heart*	*Lung*	*Brain*	*Fa[t]*
	$+G_m$	0.93	0.96	1.10	1.26	2.08	7.76	8.95	9.55	10.59	13.2
ch	0.93					**	**	**	**	**	**
	0.96					**	**	**	**	**	**
	1.10					**	**	**	**	**	**
ne	1.26						**	**	**	**	**
	2.08						**	**	**	**	**
al	7.76										
	8.95										
	9.55										
	10.59										
	13.21										

om NEWMAN KEULS and WINER Statistical principles in experimental design, 1962
Significant at the 5% level, ** significant at the 1% level
bbreviations $+G_m$ = geometric mean, Fat = subcutaneous fat

consonance with the findings of KAZAL *et al* [7] with regard to the kidney inhibitor

Table III and IV show statistical analyses of the inhibitory activities of the tested tissues' ether extracts This was a test at the 2-gram equivalent concentration At this level of concentration, no significant difference was found to exist in the group comprising stomach whole kidney, liver, and intestine These aforementioned tissues, however, differed significantly from the inhibitory activity of spleen Skeletal muscle, heart, lung, brain and subcutaneous fat did not differ significantly from each other in their action against erythropoietin The ether extract of intestine at the 2-gram equivalent concentration did not differ significantly from the ether extract of spleen

Discussion

The bulk of information on erythropoietin genesis has centered on the kidney and consequently the idea of erythropoietic inhibition had come,

Acta Haemat 52 201–206 (1974)

DNA Replication of Human Acute Leukemia Cells Cultured *in vitro*[1]

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Key Words
Autoradiography
DNA synthesis
^{3}H thymidine incorporation
Leukemic cell cycle

Abstract The DNA replication patterns previously described in normal human lymphocytes cultured *in vitro* have also been observed in the peripheral blasts of acute leukemia patients In 6 ALL cases the labeling index had a mean value of 4.5% and the 3 patterns of labeling showed a distribution very similar to that found in normal lymphocytes stimulated by PHA On the contrary, in 11 AML cases the labeling index presented a mean value of 13 9% with an increase of phase I labeling pattern in most but not all cases From these data, we suggest that the DNA synthesis of peripheral blast cells is in most cases of AML much slower than in ALL.

Nonrandom distribution of ^{3}H thymidine incorporated in the interphase nuclei of cells during DNA synthesis has been previously observed by various authors [5, 7, 8, 12] In a previous paper, the DNA replication patterns of normal human lymphocytes cultured *in vitro* and stimulated by PHA were reported [9] Phase I light, homogeneous labeling on the whole nucleus and usually no labeling over the nucleolus (nucleolus-negative phase), phase II heavy labeling equally distributed over the whole nucleus and nucleoli (intermediate phase), and phase III heavy labeling over limited areas of the nucleus and sometimes over the nucleolus (nucleolus-positive phase) In those studies, we showed that phase I was the first to be observed at the beginning of DNA synthesis and that phase III was the last to be observed during the S phase The distribution of the 3 phases at the end of 72 h of normal lymphocyte cultures stimulated by

[1] This work was presented in part at the Second Meeting of the European and African Division of the International Society of Haematology, Prague, 1973

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Nonrandom distribution of ^{3}H thymidine incorporated in the interphase nuclei of cells during DNA synthesis has been previously observed by various authors [5, 7, 8, 12]. In a previous paper, the DNA replication patterns of normal human lymphocytes cultured *in vitro* and stimulated by PHA were reported [9]. Phase I: light homogeneous labeling on the whole nucleus and usually no labeling over the nucleolus (nucleolus negative phase); phase II: heavy labeling equally distributed over the whole nucleus and nucleoli (intermediate phase), and phase III: heavy labeling over limited areas of the nucleus and sometimes over the nucleolus (nucleolus positive phase). In those studies we showed that phase I was the first to be observed at the beginning of DNA synthesis and that phase III was the last to be observed during the S phase. The distribution of the 3 phases at the end of 72 h of normal lymphocyte cultures stimulated by

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PHA was phase I 17%, phase II 55% and phase III 28% of the replicating cells The purpose of the present study was to investigate the presence of DNA replication patterns on acute leukemia cells

Materials and Methods

17 patients of acute leukemia with circulating blast cells have been studied The series includes 6 cases of acute lymphatic leukemia (ALL) and 11 cases of acute myeloid leukemia (AML) 2 of them being cytologically considered acute promyelocytic and acute monoblastic leukemia (table II case 11) None of these patients had received previous therapy

10 ml of venous blood was withdrawn into a sterile tube containing 0.5 mg heparin Cells were let to sediment at 37 °C for about half an hour, and from the leukocyte rich plasma layer cultures were prepared with a final concentration of 10^6 cells/ml Culture medium 199TC Difco was supplemented with 20% AB serum ^{3}H methyl thymidine 1 µCi ml (spec act 1 9 Ci mM) was added at the beginning of the 4 hour cultures All studies were done in duplicates Kodak AR 10 stripping film was used for autoradiography with 3–4 weeks exposing time

From each duplicate 2,000 cells were reviewed for assessing the labeling index and at least 500 cells were studied for the determination of the cell phases. Only cells with more than 5 grains on the nucleus were considered labeled

Results

The labeling index of the 6 ALL patients was consistently low with a range from 0 3 to 11% and a mean value of 4.5% (table I) The 3 patterns of DNA synthesis could be observed in all cases with a mean distribution of phase I 22 5% phase II 53 6% and phase III 23 7% This distribution is very similar to that found in human lymphocytes stimulated by PHA (fig 1)

In contrast the labeling index of 11 cases of AML showed a much wider variation from 0 6 to 38% and a mean value of 13 9% (table II) The lowest values were observed in case 4 with AML, case 11 with acute monoblastic leukemia and in case 7 with a very high peripheral blast count Case 8 an acute promyelocytic leukemia had a rather high labeling index and responded very well to therapy

The phase distribution shows a surprising high percentage of labeled cells in phase I in most but not all cases studied On the contrary, phase III shows rather low percentages (fig 2) In case 4 with the lowest labeling index almost all labeled cells showed a phase I pattern Cases 8 and 9

[illegible]

[illegible]	[illegible]	[illegible]	[illegible]		[illegible]			
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
[illegible]	[illegible]	[illegible]		[illegible]		[illegible]		[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		
[illegible]	[illegible]		[illegible]	[illegible]	[illegible]			
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
				[illegible] [illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

[illegible]

[illegible] DNA [illegible]

[illegible] AML [illegible] DNA [illegible]

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Materials [illegible]

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10 ml of venous blood was withdr[illegible]
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kocyte rich plasma layer cultures w[illegible]
cells ml Culture medium 199TC D[illegible]
methyl thymidine, 1 μCi/ml (spec[illegible]
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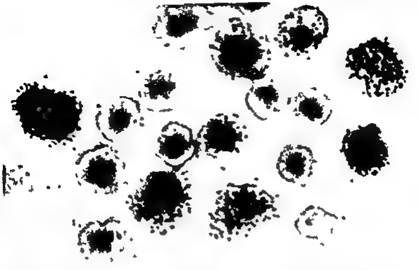

Fig 2 AML (case 10) The majority of cells incorporating ^{3}H thymidine show a phase I pattern The cell on the left presents a phase II pattern One cell on the right has incorporated more ^{3}H thymidine than the typical phase I cells and would also be considered phase II $\times$ 630

From our data, we can only suggest that the labeling index of peripheral blasts is usually lower in ALL than in AML Despite this lower labeling index, ALL cells synthesizing DNA show a much higher active process, as the majority of these cells attain phase II during the 4-hour culture Therefore, we might presume that in the peripheral blood and possibly also in the bone marrow there is a small but actively growing population of cells which might account for the better responses to chemotherapy in ALL. On the contrary, most but not all AML cases showed relatively high labeling index and the majority of AML cells synthesizing DNA presented in phase I after the 4 hour culture period Thus, although apparently there are more cells synthesizing DNA, they show a very low activity and their S period is possibly much longer Nevertheless, there are other possibilities that could explain the differences in DNA-labeling patterns observed between AML and ALL cells A decreased availability of thymidine kinase or an increased endogenous thymidine pool in AML cells could affect the amount of ^{3}H thymidine incorporated

by DNA-replicating cells [2, 10]. In summary, ALL and AML peripheral blast cells show some differences in their labeling index and distribution of their DNA replication patterns in most but not all cases studied.

Acknowledgments. This investigation was supported by a research grant from the Juan March's Foundation. I am grateful to Miss Conxa Valles and Miss Pilar Puente for their technical assistance.

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Acta Haemat 52 207–213 (1974)

Haemolysis and Erythrocyte Lipids in Thalassaemia Major

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Abstract Lipids, phospholipids and the fatty acids of phospholipid fractions were studied in the red cells of patients affected by thalassaemia major As compared to normal controls, a decrease of phosphatidylserine and phosphatidylethanolamine and of long-chain unsaturated fatty acids was detected The serum levels of tocopherols were found to be normal It is, therefore assumed that oxydation occurs in the presence of free haemoglobin chains which damage the erythrocyte membrane by binding themselves to sulfhydrylic groups

Key Words
Erythrocyte membrane
Haemolysis
Oxidation damage of red cells
Red cell lipids
Thalassaemia
Tocopherol

Among the various causes of haemolysis, possible alterations of lipids of the erythrocyte membrane have recently been taken into account [21]

In thalassaemia major, the cause of increased haemolysis has not yet been fully clarified In this disease, the red cell lipids have been studied [1, 4, 15, 17, 20] while, to our knowledge, no studies concerning the fatty acid composition of erythrocyte phospholipid fractions have been published, but only some studies pertaining to the total erythrocyte fatty acids [2, 9, 10, 18, 19]

Therefore, we have studied the red cell lipids and the fatty acid composition of phospholipid fractions in 20 patients with β thalassaemia as compared to healthy subjects of the same age

Material and Methods

Red cell lipid and phospholipid fractions were determined in 20 patients with β thalassaemia (13 males and 7 females) with an age ranging from 3 to 13 years and in 20 normal subjects (10 males 10 females) of the same age

10 ml venous blood were drawn into heparinized plastic syringe and immediately transferred to a conical test tube containing 1 mg vitamin E in order to prevent auto-oxidation. The red cells were washed 3 times with saline, suspended in an equal volume of the same and stored at −20 °C until the time for analysis (not beyond 2 weeks).

Lipid extraction was performed according to the method of DODGE and PHILLIPS [7], employing methanol containing 5% 2,6-di-*tert* butyl *p* cresol (BHT) as anti-oxidant. The lipid extract was dissolved in 1 ml chloroform, and aliquots of this solution were used for the various determinations, carried out at the same time in patient's and control's samples. Total lipids were determined with the method of BRAGDON [5], total phospholipids with the method of BAGINSKI *et al.* [3], total cholesterol with WATSON's [26] and triglycerides with FLETCHER's method [8].

Phospholipid fractions were separated by means of thin layer chromatography according to the method of SKIPSKI *et al.* [22]. 50 mg% of 2,6-di-*tert* butyl *p* cresol (BHT) were added to the solvent mixture to prevent auto-oxidation during the chromatographic run. The chromatographic spots were identified with rhodamine and removed from the plate with a spatula. Phosphorus determination was performed directly upon the silica gel with the method of BAGINSKI *et al.* [3].

Fatty acids were also determined by gas chromatography in the single erythrocyte phospholipid fractions separated by means of thin layer chromatography according to SKIPSKI *et al.* [22]. Fatty acid methylation was carried out according to JAMES *et al.* [12]. The analysis was performed with a Carlo Erba Fractovap model D gas chromatograph with a flame ionization detector and a polyester column (DEGS). The column temperature was programmed from 180 to 210 °C with an increase of 3 °C/min. Highly pure nitrogen was employed as carrier gas.

Plasma vitamin E was determined in 20 additional subjects with thalassaemia and in 20 normal controls using the colorimetric method of MARTINEK [13].

Results

The results of the quantitative determinations of red cell lipids are reported in table I. The values of total and fractioned lipids and those of the phospholipid fractions in normal subjects do not differ from those reported in the literature [7, 25, 27].

In the thalassaemic patients, there is a decrease of total phospholipids, previously pointed out by other authors [1, 4, 20], who employed methods different from ours. An analysis of the phospholipid fractions reveals a highly significant increase of sphingomyelin and phosphatidylcholine matched by a highly significant decrease of phosphatidylethanolamine and phosphatidylserine. These findings are in agreement with those of NICOLOPOULOS *et al.* [17], who used methods similar to ours and showed an increase of sphingomyelin and phosphatidylcholine. An increase of sphin-

Table I. Total and fractionated lipids (mg%/ml of packed red cells) and fractionated phospholipids (% of total phospholipids) in 20 patients affected by thalassaemia major and in 20 normal subjects

	Patients	Normals	p
Total lipids	441 ± 36	472 ± 42	0.05
Total phospholipids	248 ± 28	278 ± 39	0.02
Cholesterol	146 ± 25	156 ± 21	0.2
Triglycerides	33 ± 7	38 ± 11	0.1
Phospholipid fractions			
Sphingomyelin	34 ± 4	26 ± 4	0.001
Phosphatidylcholine	36 ± 4	28 ± 5	0.001
Phosphatidylserine	11 ± 3	14 ± 4	0.02
Phosphatidylethanolamine	18 ± 5	31 ± 9	0.001

gomyelin and a slight decrease of phosphatidylethanolamine were also pointed out by Mussini [redacted] [15].

The gas chromatographic determinations of fatty acids in the erythrocyte phospholipid fractions of thalassaemic subjects (table II) show some significant differences as compared to normal controls, consisting basically in a decrease of a few long-chain unsaturated fatty acids, such as C 20:4, C 24:1 and those after C 24:1. This pattern is present in phosphatidylcholine and less markedly in phosphatidylserine and in phosphatidyl ethanolamine. In the latter two fractions, in fact, the saturated/unsaturated ratio is increased, whereas in our normal subjects, it is below 1.

In some studies pertaining to the total erythrocyte fatty acids [9, 10, 18] a decrease of long-chain polyunsaturated fatty acids was also pointed out. It seems to us, therefore, that the phenomenon which we have described for the single fractions agrees with those findings.

Comment

The decrease of phosphatidylserine, phosphatidylethanolamine and of long-chain unsaturated fatty acids, particularly of arachidonic acid (C 20:4), are an index of oxidation of erythrocyte lipids, with a consequently greater predisposition towards haemolysis [6, 11]. Such oxidative process may occur either because of artefacts connected with the carrying out of

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Fatty acids were also determined by gas chromatography in the single erythrocyte phospholipid fractions separated by means of thin-layer chromatography according to SKIPSKI *et al.* [22]. Fatty acid methylation was carried out according to JAMES *et al.* [12]. The analysis was performed with a Carlo Erba Fractovap model D gas chromatograph with a flame ionization detector and a polyester column (DEGS). The column temperature was programmed from 180 to 210 °C with an increase of 3 °C/min. Highly pure nitrogen was employed as carrier gas.

Plasma vitamin E was determined in 20 additional subjects with thalassaemia and in 20 normal controls using the colorimetric method of MARTINEK [13].

Results

The results of the quantitative determinations of red cell lipids are reported in table I. The values of total and fractioned lipids and those of the phospholipid fractions in normal subjects do not differ from those reported in the literature [7, 25, 27].

In the thalassaemic patients there is a decrease of total phospholipids previously pointed out by other authors [1, 4, 20] who employed methods different from ours. An analysis of the phospholipid fractions reveals a highly significant increase of sphingomyelin and phosphatidylcholine matched by a highly significant decrease of phosphatidylethanolamine and phosphatidylserine. These findings are in agreement with those of NICOLOPOULOS *et al.* [17] who used methods similar to ours and showed an increase of sphingomyelin and phosphatidylcholine. An increase of sphin

Table I. Total and fractionated lipids (mg%/ml of packed red cells) and fractionated phospholipids (% of total phospholipids) in 20 patients affected by thalassaemia major and in 20 normal subjects

	Patients	Normals	p
Total lipids	441±36	472±42	0.05
Total phospholipids	248±28	278±39	0.02
Cholesterol	146±25	156±21	0.2
Triglycerides	33±7	38±11	0.1
Phospholipid fractions			
Sphingomyelin	34±4	26±4	0.001
Phosphatidylcholine	36±4	28±5	0.001
Phosphatidylserine	11±3	14±4	0.02
Phosphatidylethanolamine	18±5	31±9	0.001

gomyelin and a slight decrease of phosphatidylethanolamine were also pointed out by MUSSINI *et al.* [15].

The gas chromatographic determinations of fatty acids in the erythrocyte phospholipid fractions of thalassaemic subjects (table II) show some significant differences as compared to normal controls, consisting basically in a decrease of a few long-chain unsaturated fatty acids, such as C 20:4, C 24:1 and those after C 24:1. This pattern is present in phosphatidylcholine and less markedly in phosphatidylserine and in phosphatidylethanolamine. In the latter two fractions, in fact, the saturated/unsaturated ratio is increased, whereas in our normal subjects, it is below 1.

In some studies pertaining to the total erythrocyte fatty acids [9, 10, 18], a decrease of long-chain polyunsaturated fatty acids was also pointed out. It seems to us, therefore, that the phenomenon which we have described for the single fractions agrees with those findings.

Comment

The decrease of phosphatidylserine, phosphatidylethanolamine and of long-chain unsaturated fatty acids, particularly of arachidonic acid (C 20:4) are an index of oxidation of erythrocyte lipids, with a consequently greater predisposition towards haemolysis [6, 11]. Such oxidative process may occur either because of artefacts connected with the carrying out of

10 ml venous blood were drawn into heparinized plastic syringe and immediately transferred to a conical test tube containing 1 mg vitamin E in order to prevent auto-oxidation. The red cells were washed 3 times with saline, suspended in an equal volume of the same and stored at −20 °C until the time for analysis (not beyond 2 weeks).

Lipid extraction was performed according to the method of DODGE and PHILLIPS [7] employing methanol containing 5% 2,6-di-*tert*-butyl-*p*-cresol (BHT) as anti-oxidant. The lipid extract was dissolved in 1 ml chloroform and aliquots of this solution were used for the various determinations, carried out at the same time in patients and controls samples. Total lipids were determined with the method of BRAGDON [5], total phospholipids with the method of BACZYNSKI *et al.* [3], total cholesterol with WATSON's [26] and triglycerides with FLETCHER's method [8].

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tempt would also be warranted in thalassaemia major with the employment of substances yielding sulfhydrylic groups

Acknowledgement We are grateful to Miss OLGA MANNARINO for her technical aid

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Acta Haemat. 52: 214–219 (1974)

Immunothérapie non spécifique de la maladie de Hodgkin par BCG

Résultats préliminaires d'un essai contrôlé

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Abstract. 30 patients with Hodgkin's disease exhibiting symptoms with bad prognosis were randomized after obtaining a complete remission with treatment that included radiotherapy and chemotherapy. The first group received BCG immunostimulation and the second no treatment. Preliminary results indicate a better remission maintenance rate in the treated group but this improvement is not yet significant.

Key Words
BCG
Hodgkin's disease
Immunotherapy

Le développement prudent mais prometteur de l'immunothérapie dans le traitement des cancers, en particulier des hémopathies malignes, l'antigénicité probablement forte des cellules malignes de la maladie de Hodgkin [5], des essais positifs sur le lymphome des souris SJL/J, qui constitue un modèle certainement imparfait mais intéressant de la maladie humaine [3, 4]: ce sont là autant de raisons qui nous ont incités à réaliser un essai contrôlé d'immunothérapie non spécifique par BCG dans le traitement de cette maladie. Nous en présentons ici les résultats préliminaires.

Malades et méthodes

Malades. Du 1er août 1969 au 31 janvier 1973 30 malades ont été inclus dans cet essai. Il s'agissait dans tous les cas, de par le protocole de l'essai, de malades de moins de 65 ans, pour lesquels le diagnostic de maladie de Hodgkin avait pu

etre porté et précisé selon la nomenclature actuelle [6] sur un prélèvement suffisant, qui présentaient au moins deux éléments de pronostic péjoratif (type histolo- [illegible]

Les principaux paramètres sont précisés dans le tableau I

Ce meme tableau précise la nature des traitements inducteurs mis en œuvre comprenant dans la plupart des cas une chimiothérapie associant procarbazine, vinblastine, cyclophosphamide et méthylprednisolone (PVCM) dont le bilan personnel a été présenté ailleurs [1] et une irradiation large selon la technique de Kaplan, distribuant aux territoires ganglionnaires envahis et aux territoires adjacents une dose de 3500 à 4000 rad en 4 semaines

Une fois mis en rémission complète tous les malades ont reçu, apres un mois d intervalle libre, une nouvelle chimiotherapie du même type destinée à renforcer la rémission obtenue et, à son issue, ont eté «randomisés» en deux groupes l un laissé sans traitement, l'autre soumis à une immuno-stimulation non spécifique par BCG En cas de rechute, le BCG a été interrompu et les malades ont reçu un traitement variable approprié à chaque cas

Trois malades sont exclus de l analyse globale le premier n'ayant reçu qu'une seule scarification de BCG avant de rechuter d une façon foudroyante, les deux autres attribués au groupe traité ayant abandonné leur traitement pour des raisons personnelles après quelques mois d application, ces 2 malades sont toujours suivis et restent en rémission complète à douze et vingt six mois

Tous ont été régulièrement suivis jusqu'à leur décès ou jusqu au 31 janvier 1974

Méthode de traitement Les malades du groupe traité ont été soumis dès la fin de la chimiothérapie de consolidation à des scarifications hebdomadaires avec BCG selon la technique appliquée par MATHÉ *et al* [7] qui ont démontré son utilité dans le traitement de la leucémie aigue lymphoide La taille des scarifications a éte réduite quand les réactions génerales étaient trop importantes Après 3 ans d application, 5 malades, restant en rémission complète, ont été soumis à une seconde «randomisation» après laquelle le traitement a été poursuivi chez 2 d entre-eux et interrompu chez les 3 autres

Tolerance Nous avons signalé que 2 malades avaient interrompu leur traitement mais on peut préciser qu aucune intolérance majeure n'avait motivé cette interruption

Des réactions cutanées locales et une adénite discrète satellite des zones scarifiées sont pratiquement constantes Chez un enfant de 7 ans un ganglion a évolue vers la fistulisation puis a cicatrisé avec la seule interruption momentanée des scarifications sur le membre correspondant. Chez 6 malades, les scarifications se sont accompagnées de réactions générales (céphalées, hyperthermie) marquées dans les [illegible] suivantes et il a été nécessaire de réduire la taille du territoire scarifié A *titre indicatif un malade qui avait antérieurement reçu 2 ans de vinblastine hebdo*madaire a jugé le traitement par BCG beaucoup plus gênant.

Enfin signalons que 3 malades ont présenté pendant la période de traitement une varicelle et deux zonas d évolution banale alors que dans la série témoin on note un zona banal et un zona compliqué localement.

Tableau 1 Répartition des principaux paramètres selon les deux groupes de malades

	Total	Traités	Témoins
Sexe			
Masculin	20	12	8
Féminin	7	3	4
Age			
Moyen	27,6	29	26
Extrêmes	7–49	7–49	16–39
Type histologique			
I	3	1	2
II	7	5	2
III	11	7	4
IV	6	2	4
Allure évolutive			
Lente	11	4	7
Rapide	10	7	3
Non précisée	6	4	2
Phase évolutive			
Première	15	10	5
Deuxième	7	4	3
Troisième et plus	5	1	4
Stade clinique (pour la phase considérée)			
Extension topographique			
I	1	0	1
II	16	10	6
III	7	4	3
IV	3	1	2
Atteinte splénique	6	4	2
Signes généraux			
Absents	8	3	5
Présents	19	12	7
Traitements inducteurs			
Chimiothérapie			
1 PVCM	25	15	10
2 PVCM	2	0	2
Vinblastine	9	3	6
Radiothérapie			
Localisée	3	1	2
1 Kaplan	9	5	4
Kaplan élargi	9	7	2
Double Kaplan	4	2	2
Splénectomie	2	2	0

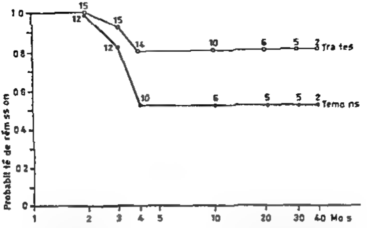

Fig 1 Courbe actuarielle de la durée des rémissions complètes Les chiffres in diquent les effectifs exposés au risque A noter que l échelle des temps est géomé trique

Résultats

La figure 1 represente la duree actuarielle des rémissions complètes à partir de la date de la «randomisation» Toutes les rechutes observees se sont manifestees dans les 4 mois suivant cette date avec par la suite une ligne de remissions complètes se maintenant horizontale et permettant de chiffrer «l espérance de guérison» Celle-ci est plus elevee pour les malades soumis au BCG (79%) que pour les témoins (53%), mais la différ ence n'est pas significative (p #0 10)

Nous avons recherche une eventuelle influence des principaux para metres representés dans le tableau I, mais n'avons trouve aucun facteur significatif

Commentaires

Les resultats actuellement enregistres ne demontrent rien Ils incitent seulement à poursuivre cet essai. Ils permettent aussi deux commen taires Sur un nombre de malades comparable, nos resultats sont beau coup moins démonstratifs que ceux obtenus par MATHÉ *et al* [7] dans le traitement de la leucémie aigue lymphoide, ce qui permet de penser qu une immuno-stimulation de ce type est a priori moins utile dans la

maladie de Hodgkin. Une immunothérapie spécifique complémentaire susceptible de renforcer l'action du BCG peut être difficilement envisagée étant donné que l'on ne peut disposer de matériel tumoral exclusif.

L'élément le plus positif qui nous paraît devoir être retenu de notre expérience est l'attitude thérapeutique plus agressive qui découle de la perspective d'une immunothérapie. Si aucun accident n'a été à déplorer dans la recherche d'une réduction tumorale maximale, il faut reconnaître que les traitements appliqués l'ont presque toujours été à la limite acceptable de la tolérance. Encore qu'aucune comparaison rigoureuse ne puisse être faite, une espérance de guérison de l'ordre de 70% pour des malades comportant en général plusieurs éléments de mauvais pronostic constitue un résultat bien supérieur à ceux retrouvés dans la plupart des séries publiées. A titre indicatif, le taux de rémissions se maintenant à 3 ans est de 63% dans l'essai H_1 concernant les stades I et II irradiés dans les conditions optimales [2]. Nous pensons, en particulier, qu'une association chimiothérapique énergique mais brève est susceptible d'accroître le taux de guérison alors qu'une monochimiothérapie au long cours ne fait que retarder la survenue de rechutes sans en réduire le taux. Malgré ses limites, notre expérience incite à développer la recherche thérapeutique, dans le cadre d'essais contrôlés, pour les maladies de Hodgkin à mauvais pronostic dans une direction jusqu'à présent relativement négligée.

Résumé

L'utilité d'une immuno-stimulation non spécifique complémentaire par BCG a été recherchée pour des maladies de Hodgkin à haut risque après réduction tumorale importante par radio et chimiothérapies. Les résultats préliminaires ne sont pas significatifs mais incitent à poursuivre cet essai s'inscrivant dans une stratégie thérapeutique sensiblement différente de celles habituellement préconisées.

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Acta Haemat. 52: 220-222 (1974)

Haemoglobin E Variants and Pregnancy in Malaysian Aborigines

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Abstract. Haemoglobin E complicates 22.2% of pregnancy in Malaysian aborigines, the prevalence of variants associated with pregnancy being: 15.8% with Hb E trait abnormality, 3.9% with Hb E homozygous disease and 2.5% with Hb E thalassaemia disease. Minor haematological abnormalities occur with the trait and homozygous conditions though a more unfavourable response is expected with Hb E thalassaemia. Haemolysis is not a prominent feature and it is suggested that factors other than the haemoglobinopathic state probably accounts for any unfavourable response in pregnancy.

Key Words: Haemoglobin E; Haemoglobinopathies; Haemolytic anaemias; Hb E thalassaemia; Malaysia; Pregnancy

Haemoglobin E (Hb E), $\alpha_2\beta_2$ (26 glu→lys) occurs predominently in Asian countries and is an unusual occurrence in the west [2, 3, 6]. Three variants are described, namely, Hb E trait (Hb E + Hb A), Hb E homozygous disease (Hb E only), and Hb E thalassaemia (Hb E + Hb F). Haematological abnormalities are minor but variable, though Hb F thalassaemia disease is known to simulate thalassaemia major in causing severe anaemia in pregnancy [2, 4, 6].

No documented study on the occurrence of Hb E and its variants in association with pregnancy is available in the literature. This paper presents the haematological response in Hb E variants associated with pregnancy in 28 Malaysian aborigines.

Materials and Methods

Haemoglobin electrophoresis was done in 126 Malaysian aborigine women in pregnancy at the Aborigine Hospital, Gombak, Selangor, West Malaysia. Other par-

ameters studied included haemoglobin (Hb) haematocrit (PCV), mean corpuscular haemoglobin concentration (MCHC) haemoglobin F (Hb F) content and reticulocyte count. The method of haemoglobin electrophoresis was based on the method described by MARENGO-ROWE [5] The haematological response in Hb E patients were compared to normal controls with Hb A

Results and Discussion

From table I, it is obvious that the haematological abnormality is least with Hb E homozygous disease, where the mean Hb is 10 5 g%, and the prevalence of anaemia in pregnancy is 20 0% The prevalence of this variant in association with pregnancy is 3 9% AUNG-THAN-BATU and U-HLA PE [1] reported a prevalence of 1 2% in the general population in Burma LIE INJO *et al* [4] commented that in this condition, no severe *anaemia occurs and the symptomatology is mild, both clinical and haem*atological This is true as can be seen from the present study The mean Hb F content is 1 8% and the mean reticulocyte count is 2 8%

In Hb E trait, the haematological abnormality is mild, compared to Hb A The mean Hb is 9 4 g% and the prevalence of anaemia in pregnancy is 55 0% AUNG-THAN BATU and U HLA PE [1] *reported a 16 6%* prevalence of this variant in Burma, with a mean Hb of 13 9 g% The prevalence of this variant in association with pregnancy in Malaysian ab-

Table I Haematological data in Hb E variants associated with pregnancy

	Number of patients	Hb g%	PCV %	MCHC %	Hb F %	Reticulocytes %	Prevalence of anaemia (Hb<10 g%) %
Hb E trait (Hb E + Hb A)	*20* (15 8%)	*9 4*	*31 0*	*31 7*	*1 5*	*2 5*	*55 0*
Hb E homozygous disease (Hb E only)	5 (3 9%)	10 5	34 6	30 6	1 8	2 8	20 0
Hb E thalassaemia (Hb E + Hb F)	3 (2 5%)	7 8	24 7	31 0	11 1	9 3	66 7
Hb A (normal)	[illegible] (76 2%)	10 2	31 9	31 9	1 0	2 6	47 9

sell's viper venom. Therefore, the factor X Friuli is found to be low only when tissue whole or partial thromboplastin is used in the assay system [5, 6].

It was subsequently shown that prothrombin level and activity in all Friuli patients is normal [10]. Furthermore, no inhibitor or PIVKA-like substance was found in Friuli plasma [12]. Finally, it was shown that the abnormal factor X has the same cross-over electrophoretic mobility as normal factor X and a different one from the coumarin induced abnormal factor X [11, 15].

The patients homozygote for the factor X Friuli coagulation disorder present a moderate bleeding tendency characterized mainly by easy bruising, menomethrorrhagias, epistaxis, bleeding after tooth extractions and after surgical procedures. Hemarthrosis was seen only in one patient after a trauma [4–6, 9, 13, 14].

During the past years we have had the opportunity to treat some of these patients and to gather some information about the management of this condition. The object of the present paper is to report some data concerning the factor X survival and the threshold factor X levels for hemostatic competence found in 4 patients with this peculiar disorder.

Materials and Methods

Materials and methods have been described in details elsewhere [5, 9]. Four patients with the factor X Friuli coagulation disorder have been investigated. They were previously reported by us in detail [6, 9, 13–16].

Patient 1 was a female (V. R.) aged 70 who was admitted to our hospital for a multiple tooth extraction on 5/3 1972. The patient weight was 53 kg and the hematocrit was 40%. She was given 4 U of Bebulin 500, namely 80 ml of factor X concentrate equivalent to 2,000 ml of normal plasma in about 15 min. The surgical procedure was carried out immediately after the end of the infusion. Only minimal bleeding was noted during the tooth extraction and no bleeding occurred therafter. No fever appeared. The only medications assumed by the patient after the procedure were a synthetic Penicillin 250 mg q.i.d. and Novalgin, a pyrazolon derivative 15 drops t.i.d. or q.i.d. for pain relief.

Patient 2 was a 32 year-old female (B. A.) who was admitted to our department on 11/13 1972 for a multiple tooth extraction too. The patient weight was 60 kg and the hematocrit was 35%. Four factor X concentrates (Bebulin 500) for a total of 80 ml equivalent to 2,000 ml of normal plasma were given and the dental procedure carried out immediately thereafter. Again bleeding was normal during the procedure and no bleeding was noted thereafter. Ampicillin 250 mg q.i.d. and Novalgin drops t.i.d. or q.i.d. for pain relief were the only medications.

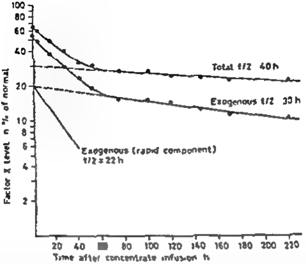

Fig 1 Factor X survival time in patient 1 (V R.) after a single infusion of 4 U of factor X concentrate (Bebulin 500) equivalent to 2 000 ml of normal plasma A multiple tooth extraction was carried out after the infusion without undue bleeding The half life of the exogenous component is 30 h

Patient 3 was a 44-year-old female (S A) who was admitted to Pordenone City hospital for hematuria on 1/1/1972 The factor X level found on admission was 12% of normal, in good agreement with previous results obtained in Padua The patient was placed on absolute bed rest and replacement therapy was begun During the first 9 days of her hospital stay she received several units of whole blood, fresh or frozen plasma and factor X concentrate (PPSB Centre National Transfusion Sanguine, Paris) and the bleeding subsided and finally disappeared after 7 days of treatment. Routine renal function studies failed to reveal any impairment

Patient 4 was a 42 year-old male (M G) who was admitted to the Pordenone City hospital for a large posttraumatic hematoma of the left zygomatic area and fracture of the zygomatic bone on 11/2/1973 During the first 5 days of his hospital stay the patient received 1 U of factor X concentrate and several 250–300 ml U of fresh plasma

Results

Factor X over all survival in patients No 1 and 2 was 40 and 38 h (fig 1, 2) The curves are practically identical and may be broken up in at least two components, a rapid and a slow one After the deduction of the basal, pretransfusion level another curve is obtained, which too may

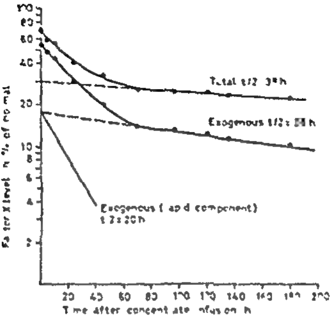

Fig. 2. Factor X survival time in patient 2 (B. A.) after the infusion of 4 U of factor X concentrate (Bebulin 400). The results are similar to those observed in patient 1.

be divided in two components, a rapid and a slow one. The half life of the exogenous or transfused component was 30 and 25 h, respectively.

The results concerning the third patient who presented hematuria are reported in figure 3. Hematuria stopped after several days of substitution therapy. The hemostatically efficient levels appeared to be around 45% of normal. During treatment, hematuria reappeared on three occasions whenever factor X was below 40% of normal but finally disappeared.

In the fourth patient we noticed a steady, even though slow improvement in the hematoma by maintaining factor X levels greater than 40% of normal over a 6-day period. Healing was satisfactory and no complications ensued.

Discussion

Our data indicate that exogenous factor X survival half life is about 30 h. Fever and hyperthyroidism are known to accelerate clotting factors turnover [22]. Our first patients did not present any of these conditions at the time of the survival studies. These data are in fair agreement with pre

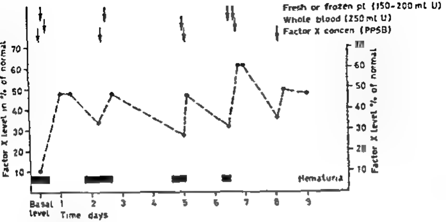

Fig 3 Factor X levels in patient 3 (S A) during multiple transfusions for persistent hematuria It may be seen that hematuria reappeared whenever factor X was below 40%

vious reports on the subject (table I) Our survival times appear to be slightly shorter than those previously reported and this could be due to the small, inevitable blood loss that occurred during the tooth extractions In this regard it is interesting to note that different half-lives have been obtained in Mr Stuart himself by different authors and on different occasions GRAHAM [19] has reported a value of 68 h whereas ROBERTS *et al* [24], 5 yers later reported survival times of 27 and 38 h after two separate transfusions

With regard to previous studies it is worth remembering also that some of the results obtained are not completely reliable in the sense that they refer to the transfusion of large quantities of plasma over a few hour period Under these circumstances approximation to the zero time becomes very difficult if possible at all

In dogs a shorter factor X half-life has been demonstrated, namely 16 5 h [20] This seems to suggest the existance of different factor X catabolic rate in other animal species as compared to man

The threshold level for bleeding manifestations in Friuli patients may be around 40–45% of normal Levels of 45–50% seem to assure adequate protection or hemostatic effeciency for tooth extractions, for the control of hematuria and for an adequate reabsorption of hematomatas This observation is in agreement with the known fact that bleeding manifesta-

Table I. Factor X survival times in congenital factor X deficiency as reported in the literature

Author	Condition	Number of patients	Number of determ.	Year	Material transfused	Eq. to ml of plasma	Half life, h	Comment
GRAHAM [19]	factor X deficiency	1	1	1960	whole plasma	1 055	65	calculated after a stabilization period of approximately 34 h
DUCKERT [3]	factor X deficiency	1		1960	unspecified		40–50	
BIGGS and DENSON [2]	factor X deficiency	1	1	1963	factor X concentrate	5 900	48	endogenous (8% of normal) component not detracted, calculated after a stabilization period of approximately 28 h
ROBERTS *et al.* [24]	factor X deficiency	2	5	1965	whole plasma	1 077 1,345	20–42 (average 31.6)	endogenous component practically equal to zero
Present study	abnormal factor X coagulation disorder	1	2	1974	factor X concentrate	2 000	28–30 (average 29)	

tions are rare and very mild among the heterozygote population in whom the factor X levels vary usually between 40 and 60% [17]

None of our patients have undergone so far major surgery and, therefore, we have no information with this regard On the basis of the experience gathered so far it would seem that such surgery could be carried out without excessive difficulty It seems, therefore, that the abnormal factor X coagulation disorder behaves as classical factor X deficiency as far as therapy is concerned This is in agreement with the known fact that no factor X inactivation occurs and that no factor X inhibitor is present in Friuli plasma The abnormal factor X in these patients, unlike the abnormal factor IX in hemophilia B_M [22], does not show any inhibitor activity [12] These facts may account for the similar behavior towards substitution therapy in classical factor X deficiency and in the factor X Friuli disorder Since the Friuli patients are not completely deficient in factor X activity it seems reasonable to expect an easier control of bleeding manifestations

A basal level of 10–14% factor X activity using a rabbit brain and lung thromboplastin has been usually found in Friuli patients This low but still present factor X activity must assure a certain hemostatic protection since the bleeding tendency presented by our patients is not severe The eldest of our patients has reached the age of 70 years without major bleeding episodes The only patient with the Friuli disorder who died prematurely (F G) presented posttransfusion hepatitis with secondary liver failure and massive hemorrhage [10] These data seem to indicate that Friuli patients have to be considered more as potential bleeders (parturition, surgery, etc) rather than actual bleeders

In classical factor X deficiency the factor X level is usually much lower (about or less than 1%) and more severe spontaneous bleeding manifestations are present Typical of this statement is the fact that no cerebral hemorrhage has ever been described in Friuli patients and no spontaneous hemarthrosis Brain hemorrhage complications have been described in classical factor X deficiency [1, 8] Mr Stuart himself has had repeated hemarthrosis of the right elbow which resulted in ankylosis [18, 19, 21]

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BIGGS and DENSON [2]	factor X deficiency	1	1	1963	factor X concentrate	5 900	48	endogenous (8% of normal) component not detracted, calculated after a stabilization period of approximately 28 h
ROBERTS *et al* [24]	factor X deficiency	2	5	1965	whole plasma	1 077 1,349	20–42 (average 31 6)	endogenous component practically equal to zero
Present study	abnormal factor X coagulation disorder	2	2	1974	factor X concentrate	2 000	28–30 (average 29)	

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Fig 1 Fluorescence pattern of a bone marrow metaphase Note the absence of an intensively fluorescing small acrocentric chromosome

Table I Summary of chromosomal findings, metaphases with 44 chromosomes had a missing Y in addition to other, inconsistent chromosome losses

Material	Date	Chromosome number			Total number of metaphases
		44	45	46	
Blood without PHA	June 14 1972	3	17	5	25
Bone marrow	July 4 1972	3	22		25
Bone marrow	January 18 1974	2	[redacted]		40
Blood with PHA	January 8 1974		[redacted]	44	50

Acta Haemat. 52: 232–237 (1974)

Ph[1]-Negative Chronic Myelocytic Leukemia with a Missing Y Chromosome[1]

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Abstract. The first case of a Ph[1]-negative chronic myelocytic leukemia with a missing Y chromosome from bone marrow cells is described. It is speculated that the benign course of the disease is due to the loss of the Y chromosome. Evidence is presented that the loss of the Y chromosome occurs on the stem cell level. No other anomalies could be detected by various banding techniques.

Key Words
Karyotype
Myelocytic leukemia
Y chromosome

It is believed that male patients with Ph[1]-positive chronic myelocytic leukemia (CML), who have a missing Y chromosome in a proportion or in all of their bone marrow cells, represent a particular subgroup of Ph[1]-positive CML [1, 5]. To our knowledge, no case with Ph[1]-negative CML and loss of the Y chromosome from bone marrow cells has been described yet. This paper presents such a case.

Case Report

The 69-year-old male patient went to his private physician in April, 1972, because of easy fatigability and a heavy feeling in his left upper abdomen. Peripheral blood and physical findings suggested CML. He was admitted to a community hospital where the diagnosis was confirmed and from where he was transferred to us in May, 1972. Physical examination revealed spleno- and hepatomegaly (10 and 3 cm below the costal margins, respectively). There was no lymphadenopathy. The patient was in good general condition. Hemoglobin 12.6 g/100 ml, platelets 153,000

[1] Supported by the Deutsche Forschungsgemeinschaft. The authors are indebted to Dr. Dornow, Lage, and Prof. Dr. Körber, Detmold, for their generous cooperation.

Fig 3 C banding patterns *a* Y negative bone marrow metaphase *b* Y positive lymphocyte metaphase The Y is indicated by an arrow

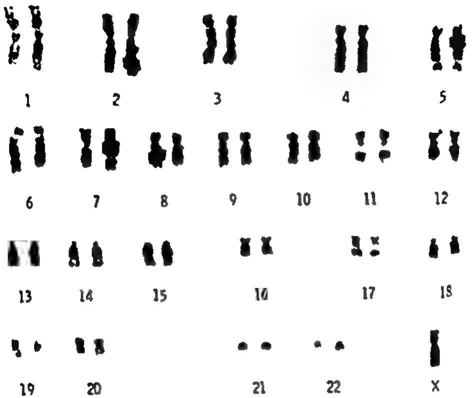

Fig. 2. Representative karyotype of a Y-negative bone marrow metaphase with a normal Q banding pattern.

mm³; white cell count (WBC) 142,000/mm³ with myeloblasts 2%, promyelocytes 4%, neutrophilic myelocytes 13%, metamyelocytes 25%, band cells 25%, neutrophilic filaments 25%, eosinophilic filaments 4%, basophilic filaments 1%, lymphocytes 1%. The bone marrow was hypercellular with a marked myelocytic hyperplasia. The proportion of immature myelocytic cells was within normal limits. Erythropoiesis was severely decreased with a myelocytic erythrocytic (M/E) ratio of 97:1. The number of megakaryocytes was normal. The leukocyte alkaline phosphatase (LAP) index was 1 (normal 15–100); vitamine B_{12} in the serum was above 5,000 pg/ml. By scintigraphy, the spleen was estimated to weight 1,033 g. It was felt that the general condition and the hematological situation of the patient did not yet require treatment with an alkylating agent. Instead, treatment with Fowler's solution was started in June, 1972, and continued by the patient's private physician until November, 1973. During this period, the patient felt well; his WBC varied between 37,000 and 140,000/mm³ with a mean of 94,500/mm³. In December, 1973, the WBC was 234,000/mm³ with myeloblasts 4%, promyelocytes 9%, myelocytes 11%, metamyelocytes 7%, band cells 29%, neutrophilic filaments 30%, eosinophilic filaments 3%, basophilic filaments 2%, normoblasts 3%, lymphocytes 2%. The spleen was firm and

mosome By this method, the polyploid metaphases, too, were disclosed to have no Y chromosome Since the polyploid metaphases are likely to be derived from megakaryocytes, and since, despite significant changes in the cellular composition of the bone marrow between May, 1972, and January, 1974, with a shift of the M/E ratio from 97 1 to 2 6 1, the chromosomal findings remained unchanged, it is postulated that all bone marrow cells – erythrocytic, myelocytic and megakaryocytic – lack the Y chromosome Thus, in this case at least, loss of the Y chromosome occurred on the stem cell level, as it is true for the induction of the Ph¹ chromosome The mechanism leading to the loss of the Y chromosome from bone marrow cells is presently unclear It may be an age-related phenomenon as suggested by PIERRE and HOAGLAND [4], which however, must have an impact on the behavior of the leukemic cells

The normal banding pattern of the bone marrow-derived metaphases as revealed by various techniques is an important unexpected observation There are to our knowledge, no reports describing the banding pattern of leukemic metaphases which appear normal with standard techniques Work is in progress in Essen to confirm this finding

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reached the left iliac crest. Treatment with busulfan was initiated, and between December 11, 1973 and January 11, 1974 the patient received a total of 184 mg. The WBC dropped to 5,000/mm³ (February 7, 1974), the spleen became unpalpable. On January 18, 1974 the marrow was normocellular in nature, the differential of the myelocytic series was normal, the M/E ratio was 2.6:1.

Chromosomal findings are summarized in table I. Marrow cells were studied on 3 occasions, PHA-stimulated peripheral blood lymphocytes once. Peripheral blood myelocytic cells, which were incubated without PHA for 20 h, were used for the first chromosome preparation. 20% of the metaphases had a normal chromosome constitution, almost 80% had a missing Y chromosome, one metaphase with 45 chromosomes had a missing C, and 3 metaphases with 44 chromosomes had inconsistent chromosome losses besides a missing Y. In the preparation of April 7, 1972 no diploid metaphases could be found; all but one showed a missing Y chromosome. On January 18, 1974, 100% of the bone marrow-derived metaphases were Y-negative, whereas about 90% of the metaphases derived from the PHA blood culture were normal. Of 8 hypodiploid metaphases in the PHA blood culture 2 had a missing Y, the others lacked different chromosomes. In none of all the metaphases studied a Ph¹ chromosome was seen.

Loss of the Y chromosome was substantiated by fluorescence (fig. 1), by G banding (fig. 2) and by C banding (fig. 3). With neither method, an abnormal banding pattern of chromosomes of bone marrow cells could be detected. In the bone marrow preparation of January 18, 1974, the polyploid metaphases were examined for the presence or absence of the Y chromosome by means of C banding: none of them had Y chromosomes.

Discussion

In our material in Essen, 9 of 96 CMLs are Ph¹-negative. Most of the Ph¹-negative CMLs have some atypical features, for instance a relatively low WBC or thrombocytopenia or high LAP. The patient presented here must be considered to have typical CML, and yet not a single metaphase contained the Ph¹ chromosome. The observation that patients with Ph¹-positive CML and a missing Y chromosome have an unusually benign course of the disease [1, 5, 6] is probably related to the loss of the Y chromosome. Hence, findings in this case allow to speculate that it is the XO constitution of the bone marrow cells which influences the development of the disease much more favorably than one usually can expect from Ph¹-negative CML in older males [2].

New techniques enable to demonstrate relatively conveniently the presence or absence of the Y chromosome in a sufficient number of metaphases. Autoradiography which we applied some years ago [3] is a much more elaborate and less reliable method. It is our experience that the C banding method is the method of choice for identification of the Y chro-

This report deals with a patient bearing the May-Hegglin anomaly whose bleeding tendency could be investigated through electron microscopy done on platelets both before and after ADP stimulation

Case Report

M A., a 28-year-old housewife was referred to our Department of Hematology because of spontaneous bleeding from her nose and gums, and easy bruisability dating since her childhood On the other hand two deliveries and a minor surgical interventation during her infancy had been uneventful Both her father and a brother had suffered from a bleeding tendency since their early years of life Moreover a hemorrhagic diathesis was also manifested by one of the two patient s daughters Except for small petechial lesions on both legs and arms her physical examination was unremarkable

The diagnosis of the May Hegglin anomaly was readily suggested by typical granulocytic and thrombocytic changes This prompted the performance of appropriate investigations All the remaining laboratory findings were within normal limits

Family investigations All members of two sibships formed by the descendants of the patient s father and by her paternal uncle were investigated by means of the platelet count and of the microscopic survey of the peripheral smear As shown in figure 1 15 out of 40 members from three generations of these two families revealed low giant platelets ranging 47 000–114 000/mm³, and typical May Hegglin leukocytic inclusion bodies

Methods

Standard methods were used for hematological studies [10] and cytochemical investigations [14 16 17 25 36 40] Platelet counts were performed with the direct method of Palumbo and Dini [32] Coagulation and hemostasis investigations were done with the methods listed in table I

For platelet separation the patient s venous blood was collected in clean plastic tubes containing one tenth volume of 3 8% trisodium citrate and then centrifuged at +4 °C for 10 min at 400 rpm in order to obtain platelet rich plasma (PRP) Platelet poor plasma (PPP) was obtained by centrifugation at +4 °C at 10 000 rpm for 15 min Adequate amounts of the patient s PRP were diluted with the PPP in order to obtain a PRP containing about 100 000 platelets/mm³

To evaluate clot retraction [27] 0 8 ml of this PRP was recalcified with 0 2 ml 2% calcium chloride and incubated for 2 h at 37 °C The platelet thromboplastin

Cross [6] according to O Brien [29] 3 ml of PRP were introduced into a clean

Acta Haemat. 52: 238-247 (1974)

The May-Hegglin Anomaly

Further Studies on Leukocyte Inclusions and Platelet Ultrastructure

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Abstract. A patient with the May-Hegglin anomaly is described with a clinical course characterized by spontaneous hemorrhages. Several studies on coagulation, hemostasis, and platelet functions did not reveal any abnormality. Cytochemical investigations were in good keeping with previous observations, and ultrastructural studies performed on platelets before and after ADP stimulation yielded normal results.

Key Words: Cytochemistry · Electron microscopy · May-Hegglin anomaly · Platelet aggregation · Platelet functions

The May-Hegglin anomaly is a rare disorder that has been a field of intensive research since its identification [18]. Thus a better understanding has been recently achieved on this hematological abnormality, such as the normal functional behaviour of the giant platelets [26] and the ultrastructure of the granulocytic inclusions [20]. Moreover, the probable difference between Dohle bodies and the May-Hegglin inclusions has been investigated recently by CAWLEY and HAYHOE [9], and the autosomal inheritance of this condition has been confirmed by SCHOLER *et al.* [37] as well as by NAFFAS *et al.* [28]. On the other hand, the reasons for which about one fourth of the reported patients suffer from a bleeding tendency are still unexplained. Indeed, this peculiar symptom did not correlate with either functional and ultrastructural platelet abnormalities in the subjects reported by LECHNER *et al.* [24]. However, in this study, electron microscopy was only done on resting platelets. Therefore, the possibility could not be excluded that some ultrastructural defects would appear following appropriate platelet stimulation.

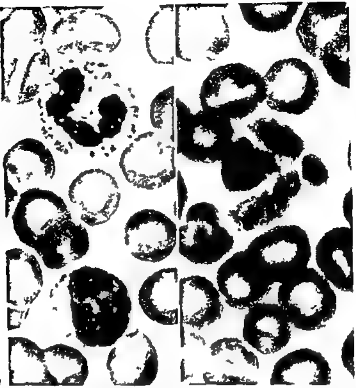

Fig 2 Peripheral blood smear May-Grunwald staining *a* A May Hegglin inclusion body in a segmented neutrophilic granulocyte *b* A giant platelet and two normal-sized thrombocytes × 830

Table II Patient M A, tests on platelet functions

Tests	Patient	Normal values	Methods
Clot retraction %	60	48–64	McFarlane [27]
Platelet thromboplastin generation test (TGT) min/sec	8/13	8/11 15	Biggs and Douglas [5]
Platelet factor 3 availability sec	23	20–25	Spaet and Cintron [38]
Platelet aggregation by ADP 0 5 µg/ml % after 5 min	56	43–65	O'Brien [29]
Platelet aggregation in silicon zed test tubes (stage)	2nd	2nd 3rd	Breddin [7]

Table I. Patient M. A. : Studies on coagulation and hemostasis

Tests	Patient	Normal values	Methods
Platelet count/mm³	55 000	200 000–400 000	PALUMBO and DINI [12]
Bleeding time, min	3	≤5	DUKE
Tourniquet test	negative	negative	
Clotting time, min	7	< 10	LEE-WHITE
Howell, sec	130	120–180	HOWELL
Partial thromboplastin time (PTT), sec	43	40–60	PROCTOR and RAPAPORT [34]
Prothrombin consumption, %	96	80–100	AGGELER *et al.* [2]
Thromboplastin generation test (TGT), min/sec			BIGGS and DOUGLAS [5]
with patient's plasma	8/10	8–12 sec	
with patient's serum	8/11	8–13 sec	
Prothrombin activity, %	84	80–120	QUICK
Factor II, %	96	60–150	OWREN and AAS [31]
Factor V, %	*110*	*50–150*	*STEFANINI [39]*
Factor VII, %	94	42–165	OWREN and AAS [31]
Factor X, %	101	50–150	DENSON [12]
Fibrinogen, mg%	350	200–400	RATNOFF and MENZIE [35]
Euglobulin lysis time, h	4	≥2–4	VON KAULLA

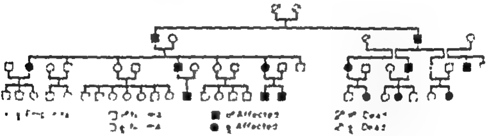

Fig. 1. Pedigree of kindred M.

plastic test tube for electron microscopic investigations and then divided into three samples. The first of these was immediately fixed at room temperature for 30 min in 1% glutaraldehyde in 0.13 M phosphate buffer (pH 7.4). The remaining two samples were fixed after 1 and 5 min, respectively, since addition of ADP in 0.13 M phosphate buffer (pH 7.4) at the final concentration of 0.5 µg/ml. After the glutaraldehyde fixation, the three samples were centrifuged at room temperature for 20 min at 2,000 rpm.

iphery, averaging 2–5 μ in diameter, and staining a sky blue color against the pinkish gray background of the neutrophilic cytoplasm (fig 2a) Cytochemical investigations showed that these bodies were strongly pyroninophilic, appearing as a well-defined area against the cytoplasmic background On the other hand, negative results were obtained for PAS, Sudan black B, both alkaline and acid phosphatases, and peroxidases The platelets varied greatly in size and shape in addition to a lesser number of normal appearing thrombocytes, most elements measured up to 11 μ in diameter and displayed bizarre forms (fig 2b) On bone marrow aspirates, the megakaryocytes were slightly increased in number Many of them were surrounded by newly formed platelets which closely resembled those observed in the peripheral blood

As shown in table I, both the coagulation and hemostasis tests afforded normal results Also, no abnormalities were disclosed by some platelet function tests, including clot retraction, platelet TGT and factor 3 availability, and platelet aggregation by either ADP or rotation in siliconized test tubes (table II) The electron microscopic investigations on resting platelets showed no ultrastructural abnormalities, other than their increased size The megathrombocytes were generally oval in shape and had intact plasma membranes and randomly distributed organelles (fig 3) Ultrastructural studies performed 1 and 5 min after ADP stimulation showed a normal aggregation of the platelets that had unaltered membranes and cytoplasmic organelles

Discussion

In this study, both the pyroninophilia and the negative cytochemical stainings of the May Hegglin inclusion bodies confirm some previous observations [20, 30, 33] However, the origin and significance of these bodies have been recently attributed to the presence of amorphous tangles of RNA within the leukocyte cytoplasm [20, 21] This hypothesis is supported by the disappearance of pyroninophilia after ribonuclease digestion [20, 21, 41] On the other hand, May-Hegglin inclusions also resemble the long recognized Dohle bodies that temporarily appear in the course of various acquired disorders [1, 19, 23, 42] Although both these leukocytic inclusions have been considered as identical by some investigators [1, 11, 13, 43] recent evidence has seriously challenged this contention Indeed, the Dohle bodies are detected exclusively within the neutro-

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phils and are both smaller [19] and less clearly defined [23] than the May Hegglin inclusions. Moreover, the former are a transient phenomenon – that is, in no instance associated with either thrombocytopenia or morphological platelet abnormalities. Also, while the May Hegglin inclusions appear on electron microscopy as ordered arrays of both rods and filaments [20], the Dohle bodies are small cytoplasmic areas containing various amount of rough endoplasmic reticulum that is most often arranged in a parallel fashion [9]. This morphological feature probably reflects an increased cell turnover throughout the myelocytic stage.

Significant bleeding has been reported in only 12 of the 44 described cases of the May Hegglin anomaly. It has been suggested that bleeding is primarily due to deficient platelet factor 3 activity rather than to thrombocytopenia [26]. Although this possibility cannot be ruled out definitely, our investigations have not provided strong arguments in its favor. Indeed, platelet factor 3 activity was not significantly depressed in our patient, whose chief complaint had been a life long bleeding tendency. On the other hand, none of the remaining tests of coagulation, hemostasis and platelet functions, including aggregation by ADP [6], differed significantly from control values for this patient, thus confirming some previous findings of GOUDEMAND *et al.* [15]; ultrastructural studies showed normal features after ADP stimulation. Therefore, no consistent data support the supposed causes for bleeding in our case of May Hegglin anomaly. However, it is not excluded that, in some other instances, platelet functional alterations are concomitant with the thrombocytopenia, as already stated by BECK and BAUMGARTNER [3]. It is possible that some enlightment will come from a better understanding of the genetic background which corresponds to a bleeding phenotype. Although a minor chromosome abnormality could be detected in a family bearing the May Hegglin anomaly [8], this finding was probably unrelated to the latter, since it was also present in some family members who did not have hematological alterations. Moreover, normal chromosomes were found in both the father and son reported by LUSHER *et al.* [26]. All the reported patients with the May Hegglin anomaly have been heterozygotes. Thus, it is unknown, but very improbable, whether the homozygous state would be associated with a more severe hemorrhagic disorder. For instance, this occurs in the rare dystrophie hemorrhagipare congenitale [4], in which homozygotes have giant platelets, abnormal platelet thromboplastic function, and a severe bleeding disorder, while heterozygotes only have a lesser number of giant platelets [22]. In the family to which our patient belongs, every affected

member is a heterozygote Since the patient's father has had two wives and a total of twelve descendants, while the patient's paternal uncle has had seven sons, our kindred would probably constitute the largest among those reported to date

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Acta haemat. 52: 248–256 (1974)

Déficit en pyruvate kinase érythrocytaire accompagné d'une anémie hémolytique néonatale sévère

Etude familiale et caractérisation biochimique de l'enzyme

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Abstract. A case of severe neonatal hemolytic anemia caused by an erythrocytic pyruvate kinase deficiency is described. A comparative analysis of the hematimetric and biochemical data has been made. The purification of the enzyme in the parents and the study of its physicochemical constants excluded abnormal kinetics of pyruvate kinase. The correlation between the severity of the anemia, variations of hematimetric criteria and biochemical properties of the enzyme in patients suffering from mutation of that factor are considered in the light of results obtained and of observations already published.

Key Words
Congenital hemolytic anemias
Enzyme kinetics
Erythrocyte morphology
Erythrocyte enzymes
Pyruvate kinase deficiency

Le déficit en pyruvate kinase (PK) (EC 2.7.1.40) érythrocytaire est l'un des désordres métaboliques majeurs de la glycolyse. Par sa fréquence (environ 200 observations publiées actuellement dans le monde) et la gravité de son incidence hématologique, il occupe une place privilégiée dans la pathologie moléculaire du globule rouge. L'anomalie enzymatique est extrêmement polymorphe sur les plans clinique et hématologique. Classiquement cette enzymopathie ne s'accompagne d'aucune manifestation hématologique chez l'hétérozygote. Cependant quelques cas de déficit partiel avec retentissement hémolytique sévère ont été rapportés particulièrement chez les nouveau-nés [5, 17]. Chez les individus homozygotes la sévérité de la maladie est très variable allant de l'hémolyse bien compensée à la forme grave d'anémie hémolytique nécessitant de fréquentes transfusions sanguines [13]. Cette hétérogénéité se retrouve sur les plans biochimique et génétique.

Enfin il existe des mutations quantitatives sans anomalie des caractères cinetiques dont l'expression clinique peut être tres variée [10, 18] Dans ce travail nous rapportons une observation de ce type rencontree dans une famille d'origine locale dont la première manifestation a éte un ictère hemolytique néonatal

Observation

Marie Laure R , agee de 3 ½ mois, est hospitalisée le 4 octobre 1972 dans le Service d Hematologie infantile pour anemie sévere Il s'agit du premier enfant du couple Le pere est d origine espagnole, il a trois enfants d un premier lit qui sont tous en bonne santé La grand mere maternelle a eu 24 grossesses mais seulement 4 enfants vivants Nous n avons pu obtenir aucune précision étiologique Mais une origine hematologique n a jamais été évoquee

L'accouchement s est déroule normalement apres une grossesse de 8 ½ mois Le poids de naissance est de 2500 g Elle presente dans les 48 premieres heures un ictère d allure physiologique qui persiste 3 jours L evolution semble satisfaisante mais à l occasion d une visite médicale systématique on remarque une importante pâleur cutanéo-muqueuse Les examens biologiques témoignent de la gravité de l anémie elle est hospitalisée L etat general parait alors assez bien conserve poids 4460 g ($-2\ \sigma$) taille 58 5 cm ($2\ \sigma$), temperature normale L'examen décèle essentiellement une splénomégalie, le foie est de volume normal Il n existe aucun signe infectieux ni hémorragique Les premiers examens complementaires fournissent les resultats suivants

GR 1890000/µl, Hb 6 5 g/100 ml VMC 107 μm^3, CGMHb 32%, réticulocytes 850000/µl (45%) avec d importantes alterations de la morphologie erythrocytaire GB 29000/µl avec une formule leucocytaire normale et presence d érythroblastes Plaquettes 200000/µl La moelle a une densité cellulaire normale avec 50% d érythroblastes Bilirubine totale 26 mg/l dont 22 mg/l de bilirubine indirecte Fer sérique 252 µg/100 ml avec une capacite totale de fixation à 384 µg% Transaminases (TGO) 34 UI LDH serique 954 UI Groupe sanguin O Rh , test de Coombs direct négatif L'etude de la résistance globulaire aux solutions hypotoniques revele la présence d une double population l une majoritaire, est normale l autre est nettement augmentee Hb F 17,5% et Hb A_2 1,7% L'electrophorese de l hemoglobine des parents est normale

Materiel et methodes

Dosages enzymatiques Le sang est recueilli sur solution conservatrice ACD Cinq activités enzymatiques érythrocytaires sont évaluées chez les parents et l enfant hexokinase (HK), glucose-6-phosphate-deshydrogenase (G-6P D), lactate deshydrogenase (LDH) PK selon la technique de CARTIER *et al* [9] et glutathion reductase (GSSG R) selon la technique de HORN [11]

Purification de la pyruvate kinase Immédiatement après le prélevement de 140 ml de sang recueilli sur héparine seche et à 4°C les hematies sont lavées en KCl isotonique et

soigneusement débarrassées du plasma et de la couche leucocyto-plaquettaire. Au volume de culot globulaire sont additionnés deux volumes d'eau distillée. Les stromas sont séparés par centrifugation. La préparation de la PK semi purifiée est celle de CARTIER *et al* [10].

La morphologie érythrocytaire est étudiée sur étalements de sang capillaire colorés selon la méthode de May-Grünwald Giemsa.

Résultats

Les résultats des dosages enzymatiques érythrocytaires figurent dans le tableau I. Chez l'enfant les taux de G-6P D, de GSSG-R et de HK sont augmentes tandis que celui de la PK est réduit à 37 % de la normale, diminution compatible avec la nature homozygote de l'anomalie. Chez les parents, le taux de HK est normal, les taux de G-6P-D, GSSG-R, LDH élevés et celui de la PK abaissé. L'activité résiduelle est pour le père de l'ordre de 75 % et pour la mère de l'ordre de 50 % ce qui laisse supposer chez eux l'existence de l anomalie à l'état hétérozygote.

Caractérisation de la PK semi purifiée. Etant donné le jeune âge de la malade et la quantité de sang necessaire pour cette étude, les propriétés de l'enzyme n'ont pu être déterminées chez elle. L'analyse cinétique à partir de la préparation purifiée n'a été realisée que chez les parents. Un lot de 15 adultes normaux étudiés dans les mêmes conditions a servi de références.

1) Courbe de vitesse de reaction en fonction de la concentration en substrat et affinite de l'enzyme pour son substrat, le phospho-énol pyruvate (PEP): l'affinité d'une enzyme michaelienne (non allostérique) pour son substrat est mesurée par l inverse de la constante de Michaelis (K_m). L'affinite d'une enzyme allostérique pour son substrat est mesuree par l inverse de la constante de demi activation vis-à-vis du substrat ($K_{1/2}$). Chez les témoins la

Tableau I. Activites des enzymes erythrocytaires exprimées en micromoles de substrat transformées par millilitre de globules rouges et par minute a 25 C.

Sujets	Pyruvate kinase	Hexokinase	Glucose-6-P-deshydrogénase	Glutathion réductase	Lactate deshydrogénase
Témoins	2,44 ± 0,34	0,195 ± 0,064	1,209 ± 0,172	0,557 ± 0,124	21,276 ± 4,27
Pere	1,76 1,90	0,251	1,63	0,88	35,96
Mère	1,24 1,30	0,226	1,58	0,72	31,71
Propositus	0,92	0,309	2,31	1,0	

courbe de vitesse de reaction v en fonction de la concentration en substrat s est plus ou moins sigmoidale selon le sujet traduisant un comportement plus ou moins allosterique (fig 1a)

Pour la mère la courbe est particulierement sigmoidale et presente deux segments identifiables un segment d hyperbole pour des concentrations en substrat inferieures à 20×10^{-5} M et un segment de sigmoïde pour des concentrations en substrat superieures à 20×10^{-5} M

Si l on represente dans le systeme de Hill en abscisse log s et en ordonnee

$$\log \frac{v}{V_{max} - v}$$

avec v = vitesse à la concentration s du substrat s = concentration du substrat V_{max} = vitesse maximale à saturation en substrat la courbe représentative est une droite dont la pente est le n de Hill La valeur de ce n est superieure a 1 et d autant plus elevee que l enzyme est plus allosterique Par ailleurs la valeur de log s pour laquelle $v = \frac{1}{2} V_{max}$ soit

$$\log \frac{v}{V_{max} - v} = 0$$

est egale a log $K^{1}/_{2}$

Donc graphiquement en utilisant en coordonnees logarithmiques en abscisse s et en ordonnée

$$\frac{v}{V_{max} - v}$$

cette représentation permet la determination experimentale simultanée des deux paramètres recherches la pente de la droite = n de Hill et la $K^{1}/_{2}$ ou la K_m selon le cas qui correspond à la concentration en PEP pour laquelle

$$v = \frac{V_{max}}{2}$$

donc pour

$$\frac{v}{V_{max} - v} = 1$$

Le caractère de dualité cinétique de la mere (fig. 1a) a deja [illegible] BOIVIN *et al* [4] chez certains sujets normaux. Il se tradu[illegible] de la courbe de demi activation vis à vis du PEP ([illegible] tion de Hill avec un n egal à 1 20 dans la partie [illegible]

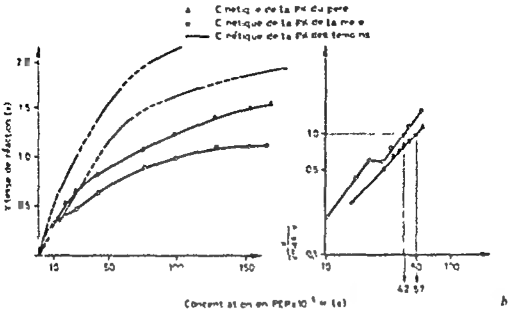

Fig. 1. Comportement de la PK purifiée. *a* Courbe de vitesse de réaction (v) en fonction de la concentration en substrat PEP (s). *b* représentation de Hill, dans un système logarithmique pour la détermination du n de Hill : pente de la droite et de $K^{1/2}$: concentration en PEP pour laquelle $v = \frac{V_{max}}{2}$ $\left(\text{ou } \frac{v}{V_{max} - v} = 1\right)$

élevé, égal à 2,00 dans la deuxième partie de la courbe. Pour le père le n est égal à 1,12. La valeur de la $K^{1/2}$ (fig. 1b) de la mère est normale : 42×10^{-5} M et celle du père subnormale : 57×10^{-5} M, nos témoins ayant donné une valeur comprise entre 35 et 50×10^{-5} M pour la $K^{1/2}$ et une valeur comprise entre 1,00 et 1,60 pour le n.

2) Comportement de la PK en présence de fructose 1-6 diphosphate (FDP). l'addition du FDP à la concentration de $3{,}5 \times 10^{-5}$ M rétablit une courbe hyperbolique dont les caractéristiques cinétiques sont identiques à celles des sujets normaux (fig. 2a). Le FDP est activateur et transforme une PK allostérique en une PK michaelienne. Il augmente l'affinité de l'enzyme pour son substrat c'est à dire qu'il diminue la valeur de la K_m (fig. 2b). Pour les témoins $K_m = 2{,}7$: 7×10^{-5} M, pour la mère $3{,}5 \times 10^{-5}$ M avec disparition normale de la double cinétique observée précédemment et pour le père $K_m = 4{,}3 \times 10^{-5}$ M.

3) Comportement de la PK en presence d'adénosine triphosphate (ATP). L'ATP accentue l'aspect allostérique de la PK en inhibant l'enzyme aussi

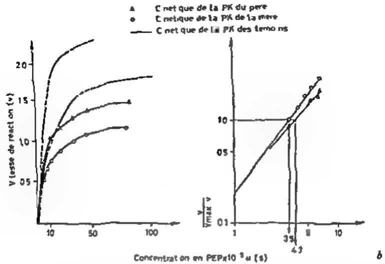

Fig 2 Comportement de la PK purifiee en présence de FDP à la concentration de 3,5 × 10^{-5} M *a* Courbe de vitesse de réaction (v) en fonction de la concentration en substrat PEP (s), *b* représentation de Hill, dans un système logarithmique pour la détermination du n de Hill pente de la droite et de K_m concentration en PEP pour laquelle $v = \frac{V_{max}}{2}$ $\left(\text{ou } \frac{v}{V_{max}\,v} = 1\right)$

bien chez les temoins que chez les parents de la malade Ce ...
tif est obtenu pour les deux concentrations d *ATP* ... en
10^{-5} M, et augmente avec la concentration de l'... inase
l'ATP deplace vers de plus fortes concentrations ...
zone de transition entre les deux types de ... c erythro-
4) Activite en fonction du pH chez les ... 28 cas avec
en fonction du pH est normale (*fig 3*) *La* ... (1972)
5,5 à pH 10,0 en tampon triethanol... pes de pyruvate
optimal se situe à pH = 7,0 comme ... maux Nouv Rev
La morphologie erythrocytaire de l'... us due to a transient
poïkilocytose, presence de schizocytes ... mat., Basel 40 166–175
hématies polychromatophiles et ...
rouges des deux parents sont ... ase in a patient with haemoly-

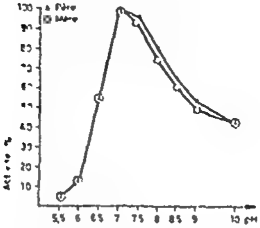

Fig. 3. Courbe pH optimal

Discussion

Depuis la découverte du déficit en PK par VALENTINE *et al.* [15] en 1961 de nombreuses observations ont été publiées [1-3, 6, 12, 14]. Plusieurs variantes de PK déficitaires ont été décrites avec bien souvent des K½ élevées. CARTIER *et al.* [10] en 1968 mettent en évidence l'interconvertibilité de deux formes de PK, allostérique et michaelienne, et suggèrent qu'il s'agit là d'une explication probable des divergences constatées. Le caractère allostérique de l'enzyme fait donc planer un doute sur les différences observées dans la littérature. Il ne peut être affirmé qu'une anomalie structurale de l'enzyme soit responsable de ces modifications cinétiques [7].

Dans notre étude, l'analyse fonctionnelle de la PK purifiée chez les deux parents ne permet pas de distinguer d'anomalie cinétique. Ceci laisse supposer que l'enfant bien qu'atteinte d'anémie grave a hérité d'une enzyme normale. Cette absence de corrélation entre le degré de sévérité clinique de l'anémie et le taux d'activité de la PK a déjà été noté par plusieurs auteurs [7, 8, 14].

Une association serait possible entre gravité des signes cliniques et troubles de la morphologie érythrocytaire. Notre proposita présentait en effet de telles anomalies. SCHROTER [13] a comparé deux cas cliniquement différents. Il a remarqué chez le patient sévèrement atteint un déficit modéré en PK mais une altération importante de la membrane des globules rouges avec diminution de l'ATPase liée à cette membrane. Par contre, le deuxième patient modérément atteint malgré une chute de 90 % de l'activité pyruvate

kinasique montrait une bonne utilisation de l ATP et des hematies morphologiquement normales

Le dosage de la PK et la caracterisation de l enzyme purifiee ne semblent pas des critères suffisants pour apprecier la severite de la maladie La baisse d activite pyruvate kinasique n'est peut-etre que l effet secondaire d un mécanisme modifiant le niveau energetique [13] ou le taux de glutathion oxyde intracellulaire [16] Peut-etre comme Bussel [8] devrions nous parler d anemie hemolytique avec deficit en PK plutôt que d anemie hémolytique par déficit en PK Seules des analyses ulterieures et une exploration plus complète du métabolisme glycolytique et energetique de cette malade nous permettront d aboutir à des conclusions definitives

Resume

Les auteurs rapportent l observation d un cas d anemie hemolytique néonatale sévere avec deficit en PK érythrocytaire Les donnees hématimétriques et enzymologiques ont éte comparees Chez les parents la purification de l enzyme et l'étude de ses constantes cinétiques ont permis d eliminer une PK à cinétique anormale Les corrélations entre gravite de l aném e variations des données hématimétriques et proprietés biochimiques de l enzyme chez les malades atteints de mutation de ce facteur sont discutees à la lumière de nos résultats et des observations publiees dans la littérature

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Acta haemat. 52 257–264 (1974)

Proliferation of Erythroblasts in Refractory Anaemia

A Combined Autoradiographic and Cytophotometric Study[1]

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Abstract. Combined cytophotometric autoradiographic investigations of erythropoiesis in refractory anaemia with hypercellular bone marrow showed a decreased percentage of DNA synthetizing basophilic and polychromatic erythroblasts with an increase of the percentage in G_1. In some cases an additional increase of the percentage in G_2 respectively a decreased S/G_2 ratio was found. The disturbance of cell proliferation is one factor leading to an ineffective cell production but was not found in 3 of the 10 patients with refractory anaemia.

Key Words
Autoradiography
Cytophotometry
DNA synthesis
Erythropoiesis
Preleukaemia
Refractory anaemia

Anaemia of unknown etiology is often associated with neutropenia and/or thrombocytopenia. The cellularity of bone marrow smears and biopsies can be normal or increased. In these cases of panmyelopathy or refractory anaemia with hypercellular bone marrow, erythropoiesis can be hyperplastic, although severe anaemia is existent. It therefore was of interest to prove, whether or not a disturbed erythropoietic cell proliferation could be responsible for the development of anaemia. The method used in this study was the combined cytophotometric autoradiographic method after *in vitro* labelling with tritiated thymidine. The distribution of proliferating erythroblasts in the various stages of cell cycle was determined.

[1] Supported by Bertha Heilscher-Stiftung.
[2] We wish to thank Miss M. BROSZIES for her excellent technical assistance.

Methods

Single cell suspensions of bone marrow were incubated for 1 h at room temperature with tritiated thymidine. The concentration of ^3H thymidine was 2 μCi/ml, the specific activity 5 Ci/mmol. Cell smears were fixed with absolute methanol (2 × 10 min) and stained by the May-Grünwald-Giemsa method. Basophilic, polychromatic and oxyphilic erythroblasts were photographed and the cell areas marked with an 'Objektmarker' (Leitz) on a second slide. Erythroblasts were classified according to Killmann [9]. The May-Grünwald-Giemsa stain was then leached out with 5% trichloroacetic acid (30 sec) and absolute methanol (10 min); the smears were then restained by the Feulgen method using a 9-min hydrolysis in 1 N HCl at 60 °C. The DNA content was measured with a cytophotometer UMSP I (Zeiss) at 560 nm. The medium DNA value of at least 40 lymphocytes represented the diploid value.

Autoradiographs were prepared by the dipping method at 18 °C, using the emulsion K5 (Ilford). Unstained smears were exposed at 2–4 °C for 7 days, smears stained by the Feulgen method for 21 days and developed for 3 min with Kodak D 19 at 18 °C. Unlabelled cells with a diploid DNA content were in the presynthetic phase (G_1), all labelled cells were in the S-phase. Unlabelled cells with a tetraploid content of DNA could be found in G_2 or premitotic phase.

Patients

We examined the erythropoiesis of 10 patients suffering from refractory anaemia with hypercellular bone marrow. Initial severe anaemia was a common symptom (table I). In 3 cases (No 3, 5, 10) neutropenia or thrombocytopenia was also present. 5 patients (No 1, 4, 6, 7, 9) suffered from pancytopenia. Leukaemia appeared 3 and 4 months after initial examination in 2 cases (No 1, 4), which implies that they were in a preleukaemic state at the time of the first examination. Megaloblastoid erythroblasts were present in 2 cases and a low percentage of ring sideroblasts in 3 cases (table II). Sideroblastic anaemias were not included in this study. The erythropoiesis of 5 healthy persons of age 20–60 was recognized as normal values.

Results

Table III shows the results of the combined cytophotometric autoradiographic study of erythropoiesis of 10 patients with refractory anaemia. It was surprising that in 3 patients (No 1–3) the distribution of proliferating erythropoietic cells, meaning basophilic erythroblasts (E_1–E_3) and early polychromatic normoblasts (E_4) in different stages of the cell cycle was normal although a severe anaemia was present. The

Table I Patients with refractory anaemia, main haematological data

Patient No	Age years	Hb g/100 ml	Neutrophils /µl	Platelets $\times 10^3$/µl	Course months	Characteristics
1	33	9 7	300	87	3	died AML
2	75	4 7	2,490	250	36	–
3	23	9.3	2,800	28	8	–
4	59	6.5	1,110	120	4	died AML
5	50	8 7	2,850	19	10	died cerebral hemorrhage
6	72	7 7	1,580	70	11	–
7	70	8.8	1,370	108	17	–
8	64	6 9	2,660	279	20	died hepatitis
9	75	9 9	841	68	5	–
10	70	9.8	1,435	395	10	–

The duration of the disease was calculated from the time of diagnosis until March 1974 or until appearance of leukaemia, respectively exitus

percentage of DNA-synthetizing basophilic erythroblasts was in 2 cases slightly higher than normal.

In another group of 5 patients, basophilic erythroblasts and early polychromatic normoblasts in G_1 were increased, while the percentage of DNA-synthetizing cells was diminished Significant quantitative differences were present among them (table III) Five patients showed an additional decreased S G_2 ratio The percentage of polychromatic or polychromatic and basophilic erythroblasts in G_2 is high in relation of the percentage of these cells in S The patient (No 10) showed a normal distribution of early polychromatic normoblasts, while the percentage of DNA synthetizing basophilic erythroblasts was decreased The disturbance of cell proliferation was recognized in the altered differential count of erythropoiesis (table IV) The percentage of the more immature forms was higher than normal, the erythropoiesis showed a 'shift to the left', which was absent in the 3 cases with normal distribution of proliferating erythroblasts in the various stages of the cell cycle

Table II. Morphological findings on erythropoiesis

Patient No	'Megaloblasts'	Ring sideroblasts	PAS reaction in erythroblasts	Morphologic abnormalities of erythropoiesis
1	ø	ø	negative	ø
2	ø	+	negative	ø
3	ø	ø	negative	[illegible]
4	+	+	negative	ø
5	+	+	negative	[illegible]
6	ø	ø	–	ø
7	ø	ø	negative	[illegible]
8	ø	ø	negative	ø
9	ø	ø	negative	ø
10	ø	ø	negative	ø

Discussion

Data in literature about cell kinetics in erythropoiesis are different. The labelling index, respectively the percentage of DNA-synthetizing basophilic erythroblasts was between 70 and 80% after *in vivo* labelling [3, 5, 9] – results which correspond with ours. Similar values after *in vitro* labelling are reported by KILLMANN [8]. Other investigators [2, 10, 13, 14] found markedly lower values. Differences were presumably caused by different techniques. The incubation time and the usage of single cell suspensions or bone marrow fragments are for the results of the *in vitro* labelling method of importance. The comparison of current results about the labelling index of early polychromatic normoblasts shows considerable variations. The lowest labelling index of 40% was given by KILLMANN [8], the highest by WICKRAMASINGHE [14] with 77–85%. Our results are comparable with the results of FLIEDNER and co-workers [3, 5, 9]. The reason for these discrepancies lies in the difficult morphological definition of early polychromatic normoblasts and especially the differentiation from the non proliferating late polychromatic normoblasts.

In 7 of 10 patients with refractory anaemia the percentage of proliferating erythroblasts in the S-phase was decreased with an increase of the percentage in G_1. This result suggests, that either the presynthetic phase is prolonged or a part of the proliferating erythroblasts do not enter the

Table III Distribution of basophilic erythroblasts (E_1–E_3) and early *polychromatic* normoblasts (E_4) in the various stages of cell cycle

Patient	E_1–E_3						E_4					
No	n	G_1	S	G_2	U	S G_2	n	G_1	S	G_2	U	S G_2
1	216	15 4	80 0	4 1	0 5	19 5	163	37 0	58 0	5 0	0	11 6
2	251	14 6	75 0	*10 4*	*0*	7 3	170	32 0	*57 5*	9 5	1 0	6 0
3	253	12 1	81 3	5 5	1 1	14 8	167	41 5	55 5	11 3	1 7	4 9
4	219	*43 5*	*47 5*	6 8	2 2	7 0	162	*61 1*	*31 5*	6 2	1 2	5 0
5	[illegible]	*28 0*	*65 0*	6 6	0 4	9 8	232	*66 5*	*26 0*	*5 0*	2 5	5 2
6	*235*	*29 4*	*68 5*	2 1	0	32 6	171	*63.2*	*33 9*	2.3	0 6	14 4
7	152	*27 0*	*64 5*	8 5	0	7 6	127	*50 0*	*40 0*	*7 0*	3 0	5 6
8	223	*24 4*	*64 1*	11 1	0 4	5 8	224	*49 0*	*30 0*	*9 3*	1 7	4 3
9	195	*26 7*	*58 6*	*11 7*	3 0	5 0	*174*	39 9	*40 0*	*17 3*	2.8	2 3
10	164	*37 0*	*60 0*	3 0	0	20 0	158	33 0	61 0	6 0	0	10 1
Normal values 5 cases	898						*917*					
Mean		16 2	75 3	6 6	<0 9	11 3		33 0	55 5	5 5	<0 9	10 0
range		11 0–22 6	73 5–77 0	3 0–13 5		5 5–24 5		28 0–47 5	46 5–66 5	5 0–6 2		7 8–10 7

U = Unknown, unlabelled cells between diploid and tetraploid value

Table II Differential counts of erythropoesis (in %)

Patient No	E_1+E_2	E_3	E_4	E_5
1	1	1.6	2.6	10.0
2	1	1.4	3.0	6.4
3	1	1.5	2.7	7.0
4	1	0.6	0.7	1.9
5	1	0.6	2.0	4.3
6	1	1.4	2.2	5.3
7	1	0.8	0.6	2.5
8	1	1.0	1.4	3.2
9	1	1.3	2.0	6.0
10	1	1.4	2.0	3.0
Normal values 5 cases	1	1.3	3.0	7.4

In each case 400 erythroblasts are differentiated

S phase. These cells presumably die or differentiate in the next cell compartment without mitosis. Similar results are obtained in studies of erythropoietic cell proliferation in 'erythroleukaemia' [5, Mitrou, Fischer and Hübner unpublished results]. Our findings indicate, that early polychromatic normoblasts are probably in part out of cycle and therefore the increased number of cells in G_1 do not indicate a prolongation of presynthetic phase. In some cases the decreased S/G_2 ratio gives evidence for a prolonged premitotic phase or an arrest of cells in G_2, which do not enter mitosis.

In studies of erythropoietic cell proliferation with *in vivo* and *in vitro* methods in diseases with erythropoietic insufficiency, similar results are obtained. The reports concern *β* thalassaemia [11, 16], congenital dyserythropoietic anaemia [10], acute leukaemia [6, 13], erythroleukaemia [5, 15], 3 cases of refractory anaemia [7], one case of 'preleukaemia' [12] and sideroblastic anaemia [15]. With exception of the inherited diseases it is a matter of erythropoietic insufficiency in leukaemia with or without hyperplastic erythropoiesis and furthermore diseases, which often represent preleukaemic states. The disturbance of cell proliferation leads to the hyperplasia of erythropoiesis. Adamson *et al.* [1] showed that in erythroleukaemia, hyperplasia and in part morphological abnormalities

of erythropoiesis are depending on erythropoietin stimulus These data suggest that disturbed cell proliferation, respectively hyperplasia are symptoms of a lesion at the stem cell level This hypothesis was supported by data obtained in experimental investigations of the 'erythroleukaemia' and acute leukaemia in mice and rats [4] The same mechanism could be responsible for bone marrow insufficiency in cases of refractory anaemia, for some of them are early stages of acute leukaemia

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Acta haemat. 52: 265–272 (1974)

In vitro Response of Bone Marrow Cells to Erythropoietin in Aplastic Anemia[1]

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Abstract. An apparent response to erythropoietin was demonstrated in all marrow cell suspensions from 12 patients with acquired aplastic anemia (8 idiopathic type and 4 drug induced aplastic anemia). However, the degree of response in aplastic anemia of both idiopathic and secondary type anemia was significantly lower than that of normal controls.

Key Words
Aplastic anemia
Bone marrow culture
Erythropoietin
Heme synthesis
Pure red cell aplasia

The pathogenesis of aplastic anemia, defective seed (stem cell) versus soil (marrow microenvironment) has recently been questioned and extensive studies have been carried out on this theme. Indirect evidence supporting defective stem cells in aplastic anemia has been accumulated, that is, the success of marrow transplants in identical twins [15], the increased incidence of nuclear abnormalities in idiopathic aplasia [13], hypoplasia as a preleukemic state [2, 4, 5], hypoplasia associated with paroxysmal nocturnal hemoglobinuria [12]. However, there is little direct evidence in favor of the defective seed theory in the pathogenesis of aplastic anemia. Recent advances in an *in vitro* culture system of marrow cells enable us to detect the abnormalities of the committed stem cells of the erythroid [9, 10] as well as granulocytic cells [3, 6]. The purpose of the present paper is to study the bone marrow response to varying doses of erythropoietin by an *in vitro* culture technique in order to elucidate the alteration of erythropoietin-sensitive cells in the marrow of aplastic anemia.

[1] Supported in part by a grant for the project investigation on pathogenesis, treatment and prevention of aplastic anemia from the Ministry of Health and Welfare, Japan.

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[illegible]

[illegible]

[illegible]

[illegible]

Table I Clinical data of 12 patients with aplastic anemia and a patient with pure red cell aplasia

No	Sex	Age, years	Etiology	Duration of illness months	Treatment	Mo
	M	[illegible]	idiopathic, acquired	1	none	
	M	50	idiopathic, acquired	12	corticosteroid hormone	8
	F	[illegible]	idiopathic, acquired	2	none	
	F	17	idiopathic, acquired	16	fluoxymesterone	[illegible]
	F	45	idiopathic, acquired	28	corticosteroid hormone	13
	M	72	idiopathic, acquired	6	none	
	F	62	idiopathic, acquired	8	fluoxymesterone	12
	F	35	idiopathic, acquired	24	none	
	F	[illegible]	drug induced, Au colloid	a) 8	corticosteroid hormone	6
				b) 12	corticosteroid hormone	10
	M	56	drug-induced, aleviatin	1	none	
	F	52	drug induced chloramphenicol	a) 1	none	
				b) 3	oxymetholone	2
	M	28	drug induced, chloramphenicol remission	12	testosterone enanthate	[illegible]
	F	41	pure red cell aplasia, acquired	a) 49	none	
				b) 51	corticosteroid hormone	1

net cpm of ^{59}Fe incorporated into heme in marrow cultures devoid of erythropoietin from controls was 138±78 (mean±SD). Marrow cells of controls showed significant increase in ^{59}Fe incorporation into heme ranging between 453 and 629% by the addition of erythropoietin. The average of maximal percentage of ^{59}Fe incorporation into heme in erythropoietin supplemented cultures was 555±167%. The dose of erythropoietin showing the maximal response of heme synthesis in controls was 0.2 U. Erythropoietin did stimulate iron incorporation into heme in marrow cells from all patients with aplastic anemia, although the net cpm of ^{59}Fe incorporated into heme in control culture of aplastic anemia marrow cells varied considerably (table II). Addition of erythropoietin up to 0.2 U/ml produced a linear increase in iron incorporation into heme in the cultured bone marrow cells from patients with aplastic anemia. In [illegible] cases, [illegible]2 U/ml of erythropoietin produced the maximal response, while in 3 cases, 0.4 U/ml of erythropoietin showed the maximal response. However, the stimulation rate of heme synthesis in the erythropoietin supplemented marrows of patients No 1–11 with aplastic anemia was significantly lower than that in the marrows of controls ($p<0.05$)

able I Clinical data of 12 patients with aplastic anemia and a patient with pure red cell aplasia

Jo	Sex	Age, years	Etiology	Duration of illness months	Treatment	Mon
	M	35	idiopathic, acquired	1	none	
	M	50	idiopathic, acquired	12	corticosteroid hormone	8
	F	61	idiopathic, acquired	2	none	
	F	17	idiopathic, acquired	16	fluoxymesterone	12
	F	45	idiopathic ,acquried	28	corticosteroid hormone	13
	M	72	idiopathic, acquired	6	none	
	F	62	idiopathic, acquired	8	fluoxymesterone	12
	F	35	idiopathic, acquired	24	none	
	F	54	drug induced Au colloid	a) 8	corticosteroid hormone	6
				b) 12	corticosteroid hormone	10
	M	56	drug induced, aleviatin	1	none	
	F	52	drug induced, chloramphenicol	a) 1	none	
				b) 3	oxymetholone	2
	M	[illegible]	drug-induced chloramphenicol remission	12	testosterone enanthate	6
	F	41	pure red cell aplasia, acquired	a) 49	none	
				b) 51	corticosteroid hormone	1

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Table II. Hematological examinations and erythropoietin responsiveness of marrow cells in patients with aplastic anaemia and pure red cell aplasia

Case No	Hb g%	Retics $\times 10^3/\mu l$	Bone marrow cellularity on aspiration	E/M ratio	Response to erythropoietin			Dose of erythropoietin at maximum response U/ml
					^{59}Fe incorporation into heme, cpm/plate		response rate maximum percent of control	
					erythropoietin supplemented culture	control		
1	10.2	17.0	D	0.88	339	94	362	0.2
2	13.0	9.7	D	0.16	200	88	228	0.2
3	8.4	16.7	D	0.36	99	59	171	0.1
4	9.9	82.0	N	1.00	1279	343	373	0.2
5	12.5	6.6	N	0.59	366	175	215	0.1
6	5.5	36.0	D	0.88	590	254	232	0.2
7	5.9	16.7	D	0.80	344	79	430	0.2
8	9.8	5.2	D	0.38	530	97	554	0.2
9	6.7	55.0	N	1.80	422	190	215	0.2
	7.5	57.0	D	0.88	970	451	215	0.4
10	7.7	24.8	D	0.73	186	38	492	0.4
11	8.6	0.0	D	0.70	91	43	210	0.2
	14.5	21.0	D	0.60	147	34	426	0.4
12	15.0	129.0	N	0.87	2970	406	730	0.2
13	7.0	0.0	D	0.0	54	56	100	–
	9.7	0.0	D	0.0	20	19	100	–

D = decreased (total cell count less than $10^5/\mu l$).
N = normal (total cell count between 10^5 and $2 \times 10^5/\mu l$).

No correlation between the degree of the marrow response to erythropoietin and the cellularity and E/M ratio of the marrow and reticulocyte count in peripheral blood was observed in all patients with aplastic anemia. Repetition of marrow cultures in case No 9 produced the same degree of maximal response to erythropoietin in both examinations, while case No 11 showed higher response in the second examination than in the first. On the other hand, case No 12 studied while in complete remission after testosterone therapy showed above-normal high level of response when erythropoietin was added. In contrast, erythropoietin did not significantly stimulate iron incorporation into heme in marrow cells from the patient with pure red cell aplasia (case No 13). The marrow

cultures from patients with polycythemia vera studied in the same way did not show any significant increase of heme synthesis by added erythropoietin in doses ranging from 0.025 to 0.40 U/ml. The net count per minute of ^{59}Fe incorporated into heme in control cultures (without erythropoietin) of these patients was 611 ± 209. The average of maximal marrow response to erythropoietin in 4 patients with polycythemia vera was $162 \pm 88\%$ (unpublished data).

Discussion

The present studies have shown that the bone marrow cells from patients with aplastic anemia contain cells capable of responding to added erythropoietin in an *in vitro* culture system. However, the degree of the response was found to be significantly lower than that obtained from normal human bone marrows. The marrow from a patient with pure red cell aplasia showed a complete lack of erythropoietin responsiveness. Although the mechanism of action of erythropoietin is not entirely clear, it is generally accepted that the erythropoietin acts on the committed stem cells (designated as erythropoietin-sensitive cells) and induces their differentiation [1, 7, 19]. STEPHENSON *et al.* [17] and STEPHENSON and AXELRAD [16] reported an assay method for erythroid colony formation from mouse fetal liver cells, but the method of *in vitro* colony formation of erythroid cell line from adult human bone marrows has not been established. The *in vitro* marrow culture system with erythropoietin has been in general use as an indirect detection procedure for erythropoietin-sensitive cells since the earlier work of KRANTZ *et al.* [9]. However, the interpretation of the data obtained from this *in vitro* culture system should be evaluated with care since it is the culture system of heterogenous cells such as bone marrow cells and the site of action of erythropoietin has not entierly been clarified.

The dose of erythropoietin providing the maximal response in marrow cultures was the same for marrows from normal controls and for marrows from patients with aplastic anemia. It seems, therefore, unlikely that the decreased response in the marrow cells of aplastic anemia was due to the diminished susceptibility of erythropoietin-sensitive cells to erythropoietin. The inhibition of heme synthesis at a higher dose of erythropoietin may occur due to the impurity of erythropoietin, as suggested by KRANTZ [10]. The possible cause for the reduced response to eryth-

ropoietin in aplastic anemia marrows is the admixture of the clone of erythroid cells unresponsive to erythropoietin as in polycythemia vera [9, 24]. But there has been no evidence for the presence of such a clone in aplastic anemia. Furthermore, another possible factor for the reduced response in aplastic anemia is an inhibitor of erythropoietin bound to the membranes of the marrow cells of patients with aplastic anemia, this hypothesis remains to be demonstrated. Since most patients with aplastic anemia in the present study had hypocellular marrows, it seems most likely that the reduced response to the erythropoietin in marrows of aplastic anemia is due to the decrease in the numbers of erythroid progenitor cells. MIZOGUCHI *et al.* [14], using the same marrow culture technique, documented two types of erythropoietin responsiveness of the marrows from their studies on 9 patients with bone marrow failure, the responsive and nonresponsive group. They also reported that all cases of nonresponsiveness eventually regained the responsiveness in remission at a later date. In our studies, there were no cases with aplastic anemia belonging to the nonresponsive group. The cases in the present study can be divided into two groups according to the degree of response, markedly decreased group and less decreased group. However, the difference of the responsiveness between these two groups may rest in the degree of marrow failure but not in qualitative change of marrow cells in each case.

Two points have to be mentioned concerning the technical problem of the employed *in vitro* culture technique. (1) the interpretation of the data obtained by *in vitro* culture using heterogenous cells such as bone marrow cells is limited. The cell-to-cell interaction among each cell line can occur and may affect the results. To avoid this, it is urgently needed to establish an efficient method to separate erythropoietin sensitive cells or *in vitro* colony formation of erythroid cell line, (2) the use of ^{59}Fe as a tracer of heme synthesis can be justified except in rare cases with advanced iron overload, since so far no specific impairment of heme synthesis has been reported in aplastic anemia [18]. It is possible that the exogeneous iron split from reticuloendothelial cells and the increased intracellular iron pool of erythroblasts causes further decrease in ^{59}Fe incorporation into heme in aplastic anemia. However, the lack of knowledge on the intracellular iron pool of erythroblasts limits the interpretation of the data from the present culture system as well. According to the results of the present studies the reduction in the number of erythropo etin-sensitive cells can be considered the most important factor in the

pathogenesis of aplastic anemia As a next step, it is necessary to determine whether the reduced number of erythropoietin-sensitive cells is due to a lack of multipotential stem cells or the damaged feed in into erythropoietin-sensitive cells from multipotential stem cells or the failure of the erythropoietin sensitive cells to differentiate into red cells Recent studies [11] and our own unpublished results on *in vitro* colonyforming cells in aplastic anemia revealed the decrease in granulocytic progenitor cells in the bone marrows of patients suffering from this disorder These results add further evidence in favor of the impairment of multipotential stem cells in aplastic anemia On the other hand, the evidence for the defective microenvironment of the marrow in aplastic anemia has recently been reported [8, 23] TRENTIN [22] and his group emphasize the importance of microenvironment for the differentiation of hematopoietic stem cells Studies along the line of defective soil and on the interaction between the stem cells and marrow microenvironment are required in further search for the pathogenesis of aplastic anemia

Acknowledgement The authors would like to express their appreciation to Dr HIBINO Director of Nagoya National Hospital for his advice and encouragement to our present studies and also to Drs Y MIURA and H MIZOGUCHI for their advice on marrow culture technique

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A Factor Causing Enhanced Viability of Lymphatic Leukaemic Lymphocytes[1]

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Abstract An experimental system was designed to search for the presence of factors in the serum of patients with chronic lymphatic leukaemia (CLL) which might affect the viability of the lymphocytes Washing was found to remove immunoglobulin from the surface of the lymphocytes In the presence of autologous serum or washings from the leukaemic lymphocytes cell viability was higher and RNA synthesis lower than in cultures with mixed human serum The factor maintaining cell viability could be adsorbed onto washed leukaemic cells with removal of the effect from the serum It is suggested that a factor is present in the sera which maintains the leukaemic population in the presence of an intact cellular immune capacity

Key Words
Cell surface factor
Immunoglobulins
Lymphatic leukaemia
Lymphocyte viability
Serum enhancing factor

The search for blocking factors in human neoplastic conditions has been based on assessing the effects of the patient's serum in abrogating the cytotoxic potential of their lymphocytes to the tumour cell [6] Serum blocking activity has been demonstrated in carcinoma of the colon [9], neuroblastoma [7] carcinoma of the lung, melanoma, osteosarcoma, carcinoma of the breast and retinoblastoma [8] Cytotoxic lymphocytes have been shown to be present in cultured peripheral blood of a patient with Hodgkin's disease [14]

Blocking factors have not been recorded in malignant lymphoma and their identification in chronic lymphatic leukaemia (CLL) presents special

[1] This work forms part of a Ph D thesis presented to the University of London by D J B PERERA

difficulties The neoplastic cells and the normal cells are derived from the same tissue and there is no method of physical separation of the cells present in the peripheral blood In addition their morphological differentiation is doubtful and their prolonged culture virtually impossible In CLL the peripheral blood is presumed to have a proportion of normal lymphocytes, mixed with a variable number of leukaemic lymphocytes [5] Although the humoral antibody response in this condition is abnormal [3] the *in vivo* cellular immune response appears to be intact [1] The satisfactory cell mediated function of the lymphocytes suggests that the leukaemic cell population might be protected from the activity of the normal cells by the presence of serum factors Possible corroborative evidence is the longevity of the lymphocytes *in vivo* in this condition [4] A precise interaction of the normal and leukaemic populations could not be carried out for the reasons given above, the present study was undertaken to show whether a factor which might be relevant to the prolonged viability of these cells *in vitro* could be demonstrated

Materials and Methods

Venous blood was taken from 14 patients with CLL and 2 healthy volunteers 15–20 ml were placed in a sterile bottle containing 100 units of heparin and serum was separated from a sample of clotted blood

Preparation of lymphocytes To the heparinized peripheral blood plasmagel was added in a proportion of 3:1 and the mixture incubated for 20 min at 37 °C. At the end of this period the red corpuscles were aggregated and sedimented The leucocyte rich plasma was carefully pipetted into a fresh 30-ml container and centrifuged at 1,500 rpm for 10 min In the case of CLL the supernatant plasma and plasmagel were removed and the cell button containing lymphocytes was resuspended with gentle mixing in 20 ml of Eagles basal medium Lymphocyte suspensions from the normal controls were obtained by layering the leucocyte-rich plasma plasmagel onto a reagent made up of 9% Ficoll and 35% triosil in the proportions of 60:25 parts [2] After centrifuging at 3000 rpm for 20 min lymphocytes were removed from the interface with less than 5% contamination with polymorphs The cells were washed 4 times after this procedure with two 15 min periods of incubation in Eagle's medium at 37 °C between the washes At the end of this procedure any serum factors on the surface of the lymphocytes should have been removed The cells were resuspended in Eagle's medium counted and a viability test performed by trypan blue exclusion

Cell culture 2×10^6/ml viable leucocytes were cultured in Eagle's basal medium containing 20% serum Cultures were performed in triplicate using sterile plastic tubes 10 × 50 mm and supplemented with one of the following autologous serum the serum of another leukaemic patient mixed human serum (MHS) and fetal calf

serum. Culture volume was 1 ml. The cells were cultured for 72 h at 37 °C in an atmosphere of 5% CO_2 and 95% air.

Absorption of serum factors. In five experiments packed, 4 times washed CLL cells were added to an equal volume of the patient's serum. The cells were suspended in the serum by gentle agitation and incubated for 1 h at room temperature. At the end of this period a cell free supernate was obtained by centrifugation at 1,500 rpm for 10 min. Eagle's medium containing 20% of this supernate was used in additional cultures to compare the effect with that of the other sera. The cells that had been used for this absorption were resuspended and cultured using mixed human serum.

Cell washings. The washings from the leukaemic cells were shown by immunodiffusion techniques to contain significant quantities of γ globulin, mainly IgG. A number of the washings were freeze dried and subsequently used after dialysis in place of leukaemic serum.

Assessment of cell survival and ³H uridine uptake. 18 h before the end of culture 2.5 μCi uridine-5-³H (specific activity 5 Ci/mmol) was added to the cultures in a volume of 0.1 ml. At the end of the 72 hour period of culture the cell suspensions were mixed thoroughly and 0.1 ml volumes placed on Whatman paper discs (grade 3 mm, size 1.9 cm). These discs were prepared in duplicate from each culture and were dried and washed in 20% trichloroacetic acid (TCA) at 4 °C for 20 min. The TCA was removed and the discs washed in 3% perchloric acid, methanol and ether. The discs were dried and introduced into a plastic vial containing a scintillant made up of 0.1 g dimethyl POPOP and 4.0 g PPO per litre of toluene. The radioactivity of the samples was then counted using a Packard liquid scintillation counter and the results expressed as cpm/10⁶ viable cells. Cell counts were performed on each culture using a Neubauer chamber and cell viabilities were assessed by Trypan blue exclusion after the addition of 1% trypan blue in a proportion of 2:1.

Results

Table I shows that absolute numbers of viable cells were highest when the washed cells were cultured in the patient's own serum, or in the serum of another patient with CLL, with the exception of patient 5.

Table II shows that generally the ³H uridine incorporation was lowest when the patient's cells were grown in the presence of autologous serum. Exceptions were 4 and 5 in which uptake was lowest with the serum of another leukaemic patient, while in patient 6 mixed sera gave marginally the least uptake of ³H uridine.

Sera from ... other pat... CLL

in cultures with the leukaemic sera while the RNA synthesis was lower

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Sera from six further patients with CLL were collected and compared with MHS in cultures using both washed leukaemic and normal lymphocytes (table III) The viability of the leukaemic lymphocytes was highest in cultures with the leukaemic sera while the RNA synthesis was lower

Table I. Effect of washing CLL cells and subsequent incubation with various sera (viability expressed as %)

	CLL patient								Normal	Normal
	1	2	3	4	5	6	7	8		
Patients serum	67	89	72	84	63	90	89	86	70	50
CLL serum	62	82	45	76	20	81	86	79	69	52
Mixed human serum	51	69	36	60	25	70	51	50	74	55
Fetal calf serum	56	61	29							
Absorbed serum				46	24	73	43	46		
Cells used for absorbing + mixed human serum				80	69	89	82	92		

except in 1 and 4. Normal lymphocyte controls showed no significant variation of cell viability or RNA synthesis in the presence of different sera, either normal or leukaemic.

When grown in MHS the leukaemic cells showed the least cell viability and usually the highest RNA activity. The increased viability of these cells in leukaemic serum as compared with MHS is statistically significant ($p<0.01$) as is the difference between the RNA synthesis in the two sera ($p<0.01$). This finding, although different from the results using normal lymphocytes where no variation was recorded, might only indicate that the leukaemic sera were superior in supporting the viability of leukaemic cells. For this reason specific absorption of the effect was next attempted.

Tables I and II show that following absorption with washed leukaemic lymphocytes the leukaemic serum is no longer superior to MHS. Cell viability is reduced and the content of RNA per cell is increased in comparison to culture with autologous serum and the results are similar to the findings with MHS. Conversely the washed leukaemic cells used to absorb the leukaemic serum factor when subsequently grown in the presence of MHS maintained their viability and exhibited a low RNA activity. In an attempt to demonstrate that antibody was involved in maintaining viability we treated the washed leukaemic cells with reconstituted freeze-dried washings containing immunoglobulin and possibly, antigen or an antigen antibody complex. These washings exerted a similar effect to the leukaemic sera (table IV) suggesting that the immunoglobulins present are responsible for the enhanced viability.

Table II Effect of wash ng CLL cells and subsequent incubation with various sera (cpm ^{3}H-uridine 10^{6} viable cells)

	CLL patient								Normal	Normal
	1	2	3	4	5	6	7	8		
Pat ents serum	1 705	1 366	1 34[illegible]	3 621	2 639	2 146	1 698	1 110	699	955
CLL serum	2 203	1 553	6[illegible]	2 839	2 449	2 308	1 950	1 432	555	863
Mixed human serum	3 698	2 81	4	4 915	2 512	2 110	2 960	2 693	787	824
Fetal calf serum	3 970	3	6							
Absorbed serum				4 070	2 997	2 305	3 260	2 868		
Cells u ed for absorb ng + m xed human serum				3 240	2 180	1 640	1 726	1 286		

Table III V ab l ty and RNA synthesis n the presence of leukaemic or normal serum (cpm able cells)

	1			4	5	6
CLL cells						
Leukaem c serum	5 380 (82)[1]		(90)	2,987 (90)	3,222 (84)	3,247 (62)
M xed human serum	5,240 (74)		(52)	2 864 (52)	4,986 (49)	4 963 (50)
Normal lymphocytes						
Leukaem c serum	699 (70)		90)	922 (74)	1,327 (69)	955 (50)
Mixed human serum	787 (69)	4	74)	887 (70)	1,249 (60)	824 (58)

[1] Percent viable cells

Table IV Effect of pre incubat on w th l n c serum eze-dried washings from leukaemic lymphocytes on the viab l ty of washe kaem c ce l ed after 3 days culture in MHS

Autologous leukaem c lymphocytes		Allogene c leuk mphocytes						Normal lymphocytes	
		1				3			
serum	wash ng	serum	wash n	er m	wa h n	serum	washing	serum	wash ng
83%	83%	90%	83%	71%	85%	72%	72%	64%	66%

Discussion

A possible explanation of our findings is that a serum protein factor maintains viability of CLL cells cultured *in vitro*. This factor does not appear to be specific for a particular patient and in some instances the serum from another patient is equally protective. Washing the lymphocytes free of this factor reduces viability in culture with MHS. We consider that this might be the result of healthy non-leukaemic lymphocytes which are known to be present in this condition, exerting a cytotoxic effect on the leukaemic cells, which have previously been protected in the body.

The *in vitro* evidence clearly demonstrates that immunoglobulins are removed from the surface of the leukaemic cells by the washing technique and that the viability of these cells can be specifically enhanced by the leukaemic sera. Freeze dried extracts of these washings are also able to enhance the viability of leukaemic cells since washed coated leukaemic cells are equally protected.

It is known that immunoglobulins are present on the majority of chronic lymphatic leukaemic lymphocytes [11, 16]. Recent studies have shown that these are less firmly bound than on normal B lymphocytes, suggesting that these cells might be coated with an anti-leukaemic antibody [15]. This would fit with the observations presented here which indicates that such an antibody is maintaining the viability of the leukaemic cells *in vitro*. The presence of this factor *in vivo* might suppress the feedback mechanism reducing normal immunoglobulin synthesis and causing further hyperplasia in lymphoid tissues.

The presence of increased RNA synthesis in cultures with low viabilities and no apparent serum effect is more difficult to understand. It is possible that the removal of the leukaemic serum allows the primed and potentially cytotoxic normal lymphocytes to react with leukaemic forms with the synthesis of more RNA. At least two antigenically distinct populations of cells exist in these patients and in the absence of the leukaemic serum these could inter-react [to be published].

Many workers have demonstrated the increased synthesis of RNA in CLL cells [10, 12]. Some have commented on the persistence of high molecular weight RNA and the deficient conversion to the cytoplasmic 28s and 18s [10]. It is possible that a factor, as proposed here, might be concerned with the abnormal metabolic changes of these cells such as abnormal glycogen synthesis [13] and defective RNA metabolism [10], in addition abrogating the effect of immune cytotoxic lymphocytes.

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Acta haemat 52 280 288 (1974)

^{67}Ga Scanning in the Staging of Hodgkin's Disease

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Abstract In 23 patients affected by untreated Hodgkin's disease ^{67}Ga citrate scanning was performed as part of a clinical and pathological staging protocol A good correlation (16/23) between ^{67}Ga scans and other diagnostic procedures was observed In 5 cases there was a disagreement between clinical staging and ^{67}Ga data Difficulties in interpreting ^{67}Ga scanning arised with the evaluation of mediastinum (5 false positives) and abdominal involvement ^{67}Ga scanning seems to be a useful but complementary procedure in the staging of Hodgkin's disease

Key Words
^{67}Ga scanning
Hodgkin staging
Isotope techniques in lymphomas

Encouraging results have been achieved during the past decade in the management of Hodgkin's disease [1, 2] This progress has been made possible by the development of new therapeutic programs including high voltage radiotherapy [3, 4] or cyclic multidrug chemotherapy [5, 6], or a combination of both [7, 8] Wide experience has proven that selection of an appropriate therapeutic regimen is entirely dependent upon accurate knowledge of the extent of the disease [9, 10] Consequently, clinical and pathological staging according to the criteria adopted by the Ann Arbor conference (1971) [11] has become a very important step in the modern approach to the treatment of Hodgkin's disease

In addition to diagnostic procedures till now employed, new scintigraphic methods with tumor specific agents have been recently introduced [12] ^{67}Ga citrate is one of the most reliable compounds for this kind of isotopic approach [13] After Ga citrate is injected intravenously, it binds with transferrin and accumulates in the kidney, liver, spleen axial skeleton and around the large joints It is excreted during the first

Fig 1 Whole body [67]Ga scan Hodgkin's disease stage IIIB

day mainly by the kidneys and then through the stools [13–16] 48–72 h after the injection there is a decrease of radioactivity in the plasma and normal tissues and an increase of radioactivity in the neoplastic lesions (fig 1) [14–18] The mechanism by which [67]Ga is concentrated in soft tissue tumors is not yet well understood [19]

Tumor visualization using ^{67}Ga scanning was first reported by EDWARDS and HAYES in 1969 [20–22]. Since then many authors confirmed [27] Ga tumor-specific properties in lymphomas and other soft-tissue tumors [15, 24]. The results of the first systematic investigation of the use of ^{67}Ga scanning in the staging of Hodgkin's disease have been reported by PINSKY *et al.* [25, 26].

This paper describes the results obtained in a series of patients with Hodgkin's disease using ^{67}Ga scanning as part of a routine staging protocol.

Materials and Methods

Patients. 23 patients with untreated Hodgkin's disease examined since April 1972 at the Medical Clinic of the University of Perugia, underwent total body ^{67}Ga scanning. This was part of a staging protocol that included bipedal lymphography, exploratory laparotomy with splenectomy and multiple biopsies, according to Ann Arbor protocols. Laparotomy was not performed in the cases of stage IIIB and IV on the basis of clinical staging.

Five patients with Hodgkin's disease who had been previously treated, but not in the last 3 months, were included as a separate group. Patients who had received radiotherapy or chemotherapy during the last 3 months have been excluded from this study.

Scan technique. Carrier free ^{67}Ga obtained by Dufhar-Philips was injected (2 μCi/kg) intravenously 48–72 h before scanning. Scans were performed on a rectilinear scanner SELO DS2 fitted with a 5×3 inch detector and a multihole collimator.

Anterior projections were obtained with the patients in the supine position. Posterior and lateral scans were not performed in this study. Purgatives and cleansing enemas were administered for 2 days to remove radioactive gallium from the colon.

Results

Results of our study with ^{67}Ga scanning in untreated patients are shown in table I. Some examples of pathological scans are reported in figure 2. In this group, a satisfactory though not absolute correlation between the scan data and the results of clinical and pathological investigations was observed. In 16 out of 23 patients the staging resulting from ^{67}Ga scan data alone was the same as that carried out on the basis of clinical diagnostic procedures, and, when performed, on the basis of pathological research. Among these 16 cases, patient No. 11 is to be

Table I Correlation of ⁶⁷Ga scan with clinical and pathological staging in untreated patients

Case No	Clinical staging	⁶⁷Ga scanning	Pathological staging	Sites of erroneous involvement[1]
1	IVB	IV	–	–
2	IIIB	III	–	–
3	IIIA	III	IIIA	Fp Md
4	IIA	II	IIA	
5	IA	III	IIIA S	Fn S
6	IIIB	III	–	Fp Md
7	IIIB	III	–	
8	IVB	IV	IVB	
9	IIIB	III	–	
10	IIIB	III	–	Fp Md
11	IVA	IV	IVA	Fp Md
12	IIA	II	IIA	
13	IIIA	III	IIA	Fp Md and P M
14	IVB	IV	–	–
15	IA	III S	IIIA S	
16	IVA	IV	–	
17	IIIB	III	–	
18	IVB	IV	–	
19	IIIA	I	–	Fn P M
20	IIA	III	–	
21	IIIA	III E S	IIA	Fp S and P M
22	IVB	IV	IVB	
23	IIIB	II	IVB	Fn S and P M

[1] Fn = False negative, Fp = false positive, Md = mediastinum S = spleen, P M = para-aortic and mesenteric lymph nodes

stressed because Ga scanning showed an abnormally high uptake in the liver and in the center of the abdomen Laparotomy confirmed these results and revealed that the abdominal uptake was due to a localization of Hodgkin's disease in the intestinal wall Some radioactivity was also present in surgically removed and cleansed intestine

In 7 patients a disagreement between ⁶⁷Ga, clinical and/or pathological staging was observed In cases 13 and 21 the laparotomy findings were negative while both lymphography and Ga scan had suggested a para aortic lymph node involvement

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[illegible]

The [illegible]
[illegible] Liver [illegible]

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Acta haemat. 52 289-293 (1974)

The Fibrinolytic Enzyme System in Acute and Chronic Liver Injury

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Abstract Components of the fibrinolytic enzyme system were measured in patients with liver disease Patients with hepatic cirrhosis and chronic active hepatitis had a trend towards increased plasminogen activator activity and decreased plasminogen levels. Patients with non-cirrhotic alcoholic liver damage had decreased activator activity with elevated fibrinogen and α_1 antitrypsin levels Primary biliary cirrhosis was associated with reduced activator activity and high levels of fibrinogen α_1 antitrypsin and α_2 macroglobulin

Key Words
Alcoholic liver disease
Chronic hepatitis
Fibrinolysis
Hepatic cirrhosis
Liver diseases
Primary biliary cirrhosis

The effect of hepatic disease on fibrinolytic activity is complex There is abundant evidence that many patients with cirrhosis of the liver have increased plasminogen activator levels and decreased plasminogen concentrations, possibly due to impaired clearance of activator by the liver [4] A few studies have pointed to changes in the fibrinolytic enzyme system in other forms of hepatic disease decreased fibrinolytic activity has been reported both in patients with cholestatic jaundice [6] and in patients with hepatic metastases [12]

In the present study we have compared the major components of the fibrinolytic enzyme system in patients with hepatic cirrhosis, alcoholic liver disease, primary biliary cirrhosis and chronic active hepatitis

Methods

Plasminogen activator was measured by performing euglobulin clot lysis times essentially by the method of Nilsson and Olow [10] using a clot lysis time recor

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Table I Values for components of the fibrinolytic enzyme system in control subjects and in patients with hepatic disease mean ± SD

	Control Subjects n	Control Subjects mean age years		*Hepatic* cirrhosis n = 10	*Chronic* active hepatitis n = [illegible]	*Alcoholic* liver damage n = 9	*Primary* biliary cirrhosis n = 9
Activator, units	50	47 7	4 2 ± 1 5	4 9 ± 1 8	5 8 ± 1 5	2 6 ± 1 5	2 7 ± 1 2
Plasminogen, casein μ/ml	65	56 8	4 4 ± 0 6	3 9 ± 1 3	3 4 ± 1 0	4 4 ± 0 9	4 9 ± 0 8
Fibrinogen mg/100ml	[illegible]	56 0	373 ± 55	402 ± 102	353 ± 101	574 ± 186	438 ± 104
FR antigen μg/ml	35	46 3	5 1 ± 4 1	7 5 ± 5 3	7 8 ± 5 7	3 0 ± 1 7	4 2 ± 3 9
Activation inhibitor, units	25	53 3	3.3 ± 1 0	4 0 ± 2.6	3 8 ± 2 4	2.7 ± 1 1	2 4 ± 1 4
α_1 Antitrypsin, mg/100 ml	80	49 3	275 ± 48	379 ± 92	265 ± 124	406 ± 95	330 ± 130
α_2-Macroglobulin mg/100 ml	50	57 3	271 ± 48	399 ± 191	311 ± 95	284 ± 82	364 ± 74

from controls ($p<0\ 05$) The mean levels of both α_1-antitrypsin and α_2-macroglobulin were moderately increased the difference from the control subjects being significant ($p<0\ 001$) in both cases

An additional patient was found at necropsy to have a hepatoma together with portal cirrhosis the plasminogen level was reduced at 2 0 μ/ml, but in contrast to the findings in uncomplicated cirrhosis, the activator level was low (under 1 0 units) and the activation inhibitor was markedly increased (over 10 units)

Chronic active hepatitis The pattern of results of the 6 patients showing the features of chronic active hepatitis on liver biopsy were similar to those found in established cirrhosis increased activator and reduced plasminogen The concentrations of fibrinogen, FR antigen, activation inhibitor, α_1 antitrypsin and α_2 macroglobulin did not differ significantly from age matched controls

Alcoholic liver disease In contrast to the findings in patients with cirrhosis and chronic active hepatitis, patients with alcoholic liver damage had decreased activator activity ($p<0\ 005$) and plasminogen levels in the normal range Both fibrinogen and α_1 antitrypsin levels were substantially elevated ($p<0\ 001$) The single patient with histological features of alcoholic hepatitis had a decreased plasminogen level (2 7 μ/ml)

Primary biliary cirrhosis The 9 patients with primary biliary cirrhosis also had a mean activator level which was significantly lower than that of

control subjects ($p<0.01$) The mean fibrinogen, α_1-antitrypsin and α_2-macroglobulin levels were all significantly elevated In this small series, the plasminogen activator level was not related to either the bilirubin or alkaline phosphatase levels ($r = +0.06$ and $+0.11$, respectively, $p<0.1$)

Discussion

A number of studies have confirmed that blood fibrinolytic activity is frequently increased in patients with hepatic cirrhosis [5, 7, 14], and that the plasma plasminogen concentration is often reduced [4, 12, 13] This combination of changes was also present in the small series of patients reported here, but they were quantitatively small It is clear that quantitative differences may result from differences in severity while the aetiology and pathological variety of cirrhosis may also be important The finding of low fibrinolytic activity in a patient who was subsequently found to have complicating hepatoma il ... trates a further reason for the hetero-
geneity of findings in respec ... fibrinolytic system in patients with
cirrhosis It is notable that t ... changes ... reduced plasminogen
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active hepatitis, a condition fr ... to

T ... spectrum of alcoholic ... inclu ... iltration of the
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c ... central hyaline ... is widely ac-
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Acta haem. 52: 294-302 (1974)

Application de la diffusion de la lumière à l'étude du volume plaquettaire[1]

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Abstract. Following a review of the basic theories of light-diffusion, the authors endeavored to apply this technique in the determination of human platelet volume. Applying Zimm's theory they succeeded *in determining the volume of light-diffusing platelets which* ranged between 3.5 and 5 μm^3. The theoretical interpretation of these results invites further investigation.

Key Words: Light scattering; Diffusion volume; *Platelet volume*; Thrombocytes; Zimm's diagram

La technique de la diffusion de la lumière a été employée pour l'étude des macromolécules [7, 15] et plus précisément pour certaines protéines [4, 5, 8, 12, 15]. De telles études peuvent fournir des renseignements quant à la masse moléculaire des produits et leur forme (sphère, bâtonnet, chaîne).

Le but de ce travail est de tenter d'appliquer cette technique non plus à des molécules, mais à des cellules biologiques (plaquettes humaines). Dans un premier temps, nous avons étudié la variation de l'intensité diffusée à un angle donné, en fonction de la concentration en cellules du milieu. Dans un second temps, connaissant cette loi de variation dans un domaine précis, nous avons essayé d'appliquer la méthode de Zimm [17] pour calculer le rayon de giration de la cellule, le facteur de forme $P(\Theta)$ et finalement un volume moyen de sphère équivalente.

Aspect théorique élémentaire

Sans entrer dans les détails, nous rappelons ci-dessous quelques aspects théoriques indispensables concernant la diffusion de la lumière. Il existe deux grands types de théories sur la diffusion de la lumière:

[1] Ce travail a été réalisé avec l'aide de la DRME (Section Biologie). Contrat no. 74.34.202.00.480.75.01.

1) Les théories concernant la diffusion par une particule unique, qui considèrent l'intensité lumineuse diffusée par la solution comme étant la somme des intensités diffusées par chaque particule Elles s'appliquent à des solutions très diluées, et ont été développées par RAYLEIGH [13], GANS [6] et MIE [11] dans le cas de particules sphériques

2) Les théories qui attribuent la diffusion à des fluctuations de concentration au sein de la solution, principalement développées par EINSTEIN et plus récemment par DEBYE [2, 3]

D'un point de vue théorique, la taille de la particule diffusante est très importante. En effet, si ses dimensions sont petites vis-à-vis de la longueur d'onde λ de la lumière incidente, elle peut être considérée comme une source lumineuse unique Par contre, lorsque ses dimensions sont comparables à la longueur d'onde, les ondes diffusées par les différents points de la particule créent des phénomènes d interférence, dont il faut tenir compte dans les calculs L'expérience montre alors que la séparation entre petites et grandes particules se fait pour $\lambda/20$

Particules de dimensions inférieures à $\lambda/20$ Pour une lumière incidente non polarisée, l'intensité I_θ de la lumière diffusée, à une distance χ de la particule et dans une direction faisant un angle θ avec la lumière incidente d'intensité I_o est donnée par la formule

$$I_\theta = \frac{I_o \times 8\pi^4\alpha^2}{\lambda^4\chi^2}(1+\cos^2\theta) \tag{1}$$

On démontre également que

$$\frac{Kc\,(1+\cos^2\theta)}{R_\theta} = \frac{1}{M} + 2Bc \tag{2}$$

$R_\theta = I_\theta\ \chi^2/I_o$ est appelé rapport de RAYLEIGH, M est la masse moléculaire de la particule, B est le second coefficient du viriel

Particules de dimensions supérieures à $\lambda/20$ Dans ce cas, on ne peut plus considérer de telles particules comme des centres diffusants uniques, et l'on observe des phénomènes d'interférence entre les rayons émis par les différents points de la particule

On définit un coefficient correctif $P(\theta)$, tel que

$$P(\theta) = \frac{\text{intensité diffusée par une grande particule}}{\text{intensité diffusée en l'absence d interférence}},$$

ou puisque les interférences sont nulles pour $\theta = 0°$,

$$P(\theta) = \frac{I_\theta}{I_{\theta = 0°}}$$

L'expression (2) devient alors

$$\frac{Kc\,(1+\cos^2\theta)}{R_\theta} = P^{-1}(\theta)\ \left[\frac{1}{M} + 2Bc\right]$$

... pour les angles faibles On a

$$P^{-1}(\theta) = 1 + \frac{16\pi^2}{3\lambda^2}\rho^2 \sin^2 \theta/2 + \varepsilon(\theta).$$

ρ = rayon de giration

Si l'on porte $P^{-1}(\theta) = f(\sin^2 \theta/2)$, la tangente à l'origine a donc une pente proportionnelle au carré du rayon de giration.

En ce qui concerne notre étude, aucune des théories émises ne semble pouvoir s'appliquer directement, car nous sommes en dehors des hypothèses de base concernant les particules diffusantes. La surface de séparation entre les particules et le milieu n'est pas définie géométriquement, car les plaquettes sont susceptibles de se déformer, de se gonfler (par échange osmotique avec le milieu) ou de se contracter. Enfin, l'indice de réfraction de la particule est mal défini. N'étant pas une particule homogène, la cellule n'a pas un indice de réfraction constant, il varie probablement du centre à la périphérie.

La théorie de MIE [11], reprise par KASTLER [9] et KERKER [10], qui semblerait la plus proche de notre cas, puisqu'elle décrit le phénomène de diffusion pour des particules sphériques de rayon quelconque et ayant des indices de réfraction même très différents de celui du milieu environnent, ne s'applique pas. En effet, nous avons effectué, sur ordinateur, les calculs selon MIE [11] pour des particules de rayon 0,8 µm et le diagramme de diffusion obtenu présente des maxima et minima successifs, que nous n'observons pas expérimentalement, puisque le diagramme de diffusion est parfaitement continu. C'est pourquoi, nous avons tenté d'approcher le volume plaquettaire par la méthode de ZIMM [17].

Materiel et Méthode

Matériel. Nous avons utilisé un photogonio-diffusemètre FICA 50, dérivé de l'étude de WIPPLER et SCHEIBLING [16]. La cellule a été modifiée pour permettre l'agitation et l'homogénéisation à l'intérieur même de la cellule sans créer de perturbation au niveau du rayon lumineux. D'autre part, des filtres de densité optique 1 et 1,5 ont été placés sur le faisceau diffusé pour atténuer l'intensité sur le photomultiplicateur.

Préparation du plasma. Le sang humain est prélevé en verrerie siliconnée, sur citrate de sodium 3,8%. Le plasma riche en plaquettes (PRP), obtenu par centrifugation lente, contient environ 200000 plaquettes/mm³. Les différentes concentrations sont obtenues par dilution du PRP par le plasma pauvre en plaquettes (PPP).

Comme le plasma contient des protéines à une forte concentration et est donc un milieu très diffusant et absorbant, il nous a paru intéressant d'étudier des suspensions de plaquettes lavées. La préparation de telles suspensions étant longue, nous nous sommes limités à vérifier que le plasma ne gênait pas l'étude de la diffusion par les plaquettes. Nous avons, en effet, obtenu les mêmes résultats lorsque l'étude par la méthode de ZIMM [17] a été effectuée sur les plaquettes en suspension dans leur propre plasma ou en suspension en eau physiologique (fig. 2, 3).

Déduction théorique. La méthode de ZIMM [17], étant de loin la plus employée pour l'étude des macromolécules, nous avons tenté d'utiliser cette technique pour étudier les suspensions de plaquettes dans le plasma. Cette technique consiste à étudier la diffusion à différentes concentrations et pour différents angles, et à porter sur un même diagramme les variations avec la concentration et avec $\sin^2 \theta/2$ de la grandeur

$$\frac{Kc(1+\cos^2\theta)}{R_\theta}$$

En extrapolant successivement à angle nul, puis à concentration nulle, on obtient la courbe $c = o$ qui est la courbe

$$\frac{P^{-1}(\theta)}{M}$$

en fonction de $\sin^2 \theta/2$, la pente à l'origine de cette courbe permet de calculer le rayon de giration de la particule

Cette technique n'a de sens que si l'extrapolation est linéaire, c'est à-dire si on travaille dans un domaine de concentration tel que l'intensité diffusée varie linéairement avec la concentration

Résultats

Notre première étude a consisté en l'étude des variations de l'intensité diffusée avec la concentration Ces variations ne sont pas linéaires mais exponentielles Nous avons été amenés à utiliser une représentation logarithmique, ainsi les courbes $\log I = f(c)$, c = (concentration en plaquettes) sont-elles linéaires (fig 1) et permettent une extrapolation Par suite, il est possible d'appliquer la methode de Zimm [17], mais en utilisant également une représentation semi-logarithmique, de façon que l'extrapolation soit linéaire (fig 2, 3)

Nous avons effectué un grand nombre de mesures sur des PRP provenant de differents donneurs, Les resultats obtenus sont très reproductibles, bien que les plasmas se présentent sous des aspects tres variés tant en ce qui concerne leur coloration que la concentration en plaquettes Nous avons d'ail-[illegible]

250000 plaquettes, la concentration est trop forte et il apparait des interactions entre les élements diffusants La courbe log I (intensité fonction de la concentration) s'incurve

Tous les diagrammes de Zimm [17] que nous avons tracés ont la même allure et sont bien reproductibles, ils conduisent tous à une courbe extrapolée à concentration nulle du même type et dont la pente à l'origine varie entre les deux bornes suivantes

$$67 < \frac{d\,(P^{-1}[\theta])}{d\,(\sin^2\,\theta/2)} < 125$$

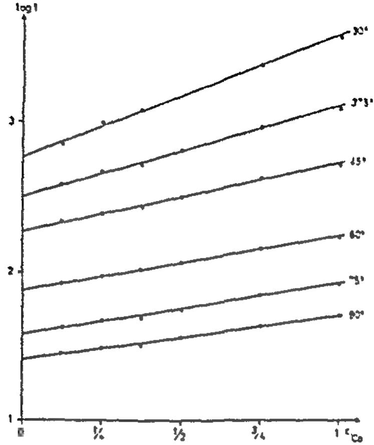

Fig. 1 Intensité diffusée par un PRP en fonction de la concentration en plaquettes.

Rappelons qu'à faible angle

$$P^{-1}(\theta) = 1 + \frac{16\pi^2}{3\lambda^2}\rho^2 \sin^2\frac{\theta}{2},$$

La pente à l'origine est donc

$$\frac{d\,(P^{-1}[\theta])}{d \sin^2 \theta/2} = \frac{16\pi^2}{3\lambda^2}\rho^2,$$

soit

$$\rho^2 = \frac{3\lambda^2}{16\pi^2} \times \text{(pente à l'origine)},$$

d'ou $0{,}614 < \rho < 0{,}841$

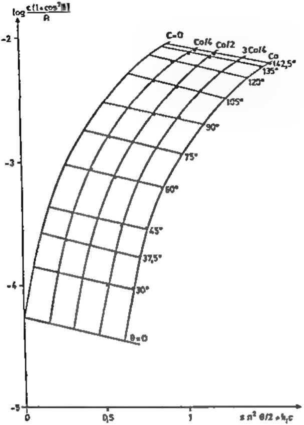

Fig 2 Diagramme de Zimm sur des suspensions de plaquettes lavées en eau physiologique

Dans l'hypothèse ou le volume diffusant est approché par une sphère, on obtient leurs rayons par la relation

$$R^2 = \frac{5}{3}\rho^2,$$

soit 0,8 μm < R < 1,1 μm, soit un volume diffusant moyen des plaquettes variant de 3,5 à 5 μm³

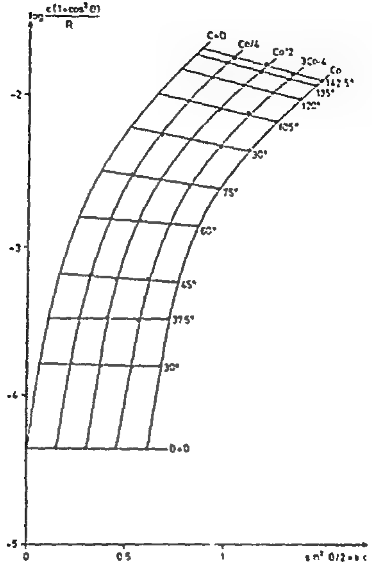

Fig. 3 Diagramme de Zimm sur des suspensions de plaquettes dans leur propre plasma.

Si, par contre, on suppose que le rayon de giration trouvé est celui d'un ellipsoïde de révolution dont le rapport grand axe sur petit axe $\frac{a}{b}$ est compris entre 2 et 3, on a

$$\rho^2 = \frac{a^2 + 2\,b^2}{5},$$

avec $a \sim 2{,}5\,b$, soit $1{,}2\ \mu m < a < 1{,}6\ \mu m$

Conclusion

La valeur du volume diffusant ainsi trouvee est proche, bien que légèrement inferieure, aux valeurs données dans la littérature Ainsi, tout laisse à penser que la technique de Zimm [17] reste valable et que le phénomène de diffusion de la lumière par le PRP, selon cette technique, concerne effectivement la diffusion par les plaquettes D'un point de vue théorique, par contre il semble difficile de justifier les résultats obtenus, en particulier les variations de l'intensite diffusée en fonction de la concentration D'autre part, les échanges cellulaires des plaquettes avec le milieu (qui tendent à modifier tant leur forme que leur volume), et l'anisotropie de ces cellules sont des facteurs dont il faudrait tenir compte dans une etude plus approfondie

Résume

Après un rappel des théories élémentaires de la diffusion de la lumière, les auteurs tentent d appliquer cette technique à la détermination du volume des plaquettes humaines Ils appliquent la théorie de Zimm, qui leur permet de déterminer le volume diffusant des plaquettes qui est trouvé compris entre 3,5 et 5 μm^3 L'interpretation théorique de ces résultats reste délicate

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Structure and Function of Haemoglobin Tacoma (β30 Arg→Ser) Found in a Second Family

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Abstract. A second finding of Hb Tacoma (β30 [B12] Arg→Ser) in a Russian patient has presented the opportunity to examine the possible involvement of Arg β30 (B12) in the alkaline Bohr effect.

Key Words
Bohr effect
Haemoglobinopathies
Hb Tacoma
Oxygen affinity of haemoglobin
Unstable haemoglobins

We have recently had the opportunity to cooperate in the study of two unstable haemoglobins found in the USSR. The first, Hb Moscva (β24 [B6] Gly→Asp), which has been described elsewhere [9] was found in a Russian woman who has since died of myeloid leukaemia. The second proved to be Hb Tacoma (β30 [B12] Arg→Ser), which has previously been described only once before in an American Caucasian family [1, 3]. Since arginine β30, the amino acid residue substituted in Hb Tacoma, is thought to be involved in the alkaline Bohr effect [13] this report presents preliminary oxygen affinity studies on the isolated haemoglobin.

Methods

Examination of the haemolysate by electrophoresis was carried out using paper [6], starch and agar gel [15, 16] and cellulose acetate [12]. The proportion of Hb F was determined by alkali denaturation [7], while Hb A_2 was quantitated both by cellulose acetate electrophoresis [12] and by DEAE Sephadex chromatography [8]. Isopropanol stability tests were carried out by the method of CARRELL and KAY [4], and heat stability tests according to DACIE *et al.* [7].

For oxygen affinity studies, Hb Tacoma was purified in the carbonmonoxy (CO-) form by chromatography on DEAE Sephadex using a Tris HCl buffer gradient [8]. The isolated CO-Hb Tacoma and CO-Hb A fractions were concentrated by ultrafiltration at 4 °C and equilibrated with 0.05 M K_2HPO_4, pH 8.6, by gel filtration on a 1×10 cm column of Sephadex G 25. The CO-haemoglobins were converted to the oxy form by illumination in the presence of oxygen according to KILMARTIN and ROSSI BERNARDI [11]. The oxygen affinity of the haemoglobins was measured by the automatic method of IMAI *et al.* [10] using 0.1 percent haemoglobin solution in 0.1 M potassium phosphate buffer of various pH values containing 0.5 mM EDTA.

For structural studies, globin was prepared from the haemolysate ('total globin') and from a 5-min isopropanol precipitate, by precipitation from acid acetone at −20 °C [21]. Cellulose acetate electrophoresis of total globin, using 6 M urea/0.025 M barbital buffer, pH 8.0 [18] was used to determine which of the chains was abnormal. The total globin was separated into its component chains by chromatography on CM-cellulose [5], the isolated chains were freed of urea and salts by dialysis into 0.5 percent (v/v) aqueous formic acid at 4 °C and recovered by freeze-drying. The unmodified α-, β^A- and β^T-chains (see results) were digested with trypsin and the soluble portion of the digests fingerprinted [19]. The region containing the variant peptide of Hb Tacoma was cut out from a preparative scale fingerprint and submitted to paper electrophoresis at pH 9 (1% v/v $[NH_4]_2CO_3$, 44 v/cm, 1 h), the purified variant peptide was located by staining with 0.02 percent (w/v) ninhydrin in acetone containing 1 percent (v/v) pyridine, eluted with 0.5 M NH_4OH, dried, and hydrolyzed in a sealed, evacuated tube with constant boiling HCl (containing 0.1 percent (w/v) phenol) [17] for 48 h at 108 °C. After removal of excess HCl on a rotary evaporator, the amino acid composition of the dried hydrolysate was determined [20] using a Locarte amino acid analyser.

Case Report

The patient suffered from pancreatitis and his haemoglobin abnormality was discovered unexpectedly during routine tests in hospital. His son, who was clinically normal, was also subsequently shown to have a heat labile haemoglobin. The isopropanol test, performed on a sample which had previously been submitted to paper electrophoresis at pH 8.9 to remove any Hb A_2, Hb A_3 and non haem proteins, was weakly positive, confirming the presence of mildly unstable haemoglobin. The Hb A_2 concentration of the patient's blood (3.7%) was very slightly raised (normal range 2.5–3.5%) while the level of Hb F (0.6%) was normal. Starch gel electrophoresis revealed a band in the region where free α-chains are normally found, and a slight smearing of the Hb A band, but no additional fraction was noted in any of the haemoglobin electrophoresis systems. Cellulose acetate electrophoresis of the globin in the presence of 6 M urea showed normal α-chains, normal β^A-chains and abnormal β^T-chains: the latter migrated more rapidly towards the anode than β^A-chains, suggesting the acquisition of one additional negative charge or the loss of one positive charge per β^T-chain.

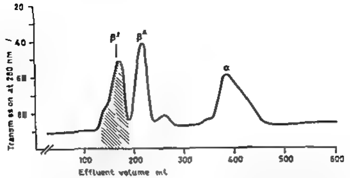

Fig 1 Chromatography of total globin using CM-cellulose and 8 M urea containing buffers pH 6 6 [5] The column (27×1 cm) was eluted at a flow rate of 60 ml/h and the eluate monitored for percent transmission at 280 nm Fractions of 10 ml were collected The cross hatched area represents the fractions containing the β^{J}-chain from Hb Tacoma

Fig 2 Fingerprint of the soluble tryptic peptides of the unmodified β-chain from Hb Tacoma Electrophoresis at pH 6 4 55 v/cm for 1 h Ascending chromatography in pyridine isoamyl alcohol water (6 6 7 by volume) for 18 h Peptides located by staining with 0 2 percent (w/v) ninhydrin in acetone (containing 1 percent (v/v) pyridine) • = origin

Results of Structural and Oxygen Affinity Studies

The elution pattern of the total globin on CM cellulose chromatography (fig 1) confirmed the presence of β^{A}-, β^{J} and α^{A}-chains the relative yields of the major chains (β^{A}, 50 mg, β^{J}, 34 mg, α^{A}, 85 mg) indicated

Table I Hb Tacoma amino acid composition of the peptide β18–40

Residue	Molar ratios	Expected for β^ATpIII + β^ATpIV
Asp	1 9 [2]	2
Thr	1 0 [1]	1
Ser	*1 0 [1]*	0
Glu	2 7 [3]	3
Pro	0 9 [1]	1
Gly	2 9 [3]	3
Ala	1 1 [1]	1
Val	4 9 [5]	5
Leu	2.7 [3]	3
Tyr	1 0 [1]	1
Arg	*0 9 [1]*	2
Trp	+	+

One amino acid residue corresponds to 32 nmol + indicates a positive staining reaction for tryptophan which is destroyed during the acid hydrolysis preceding amino acid analysis.

that the variant represented about 40% of the total haemoglobin in the patient's red cells

The tryptic fingerprint of the α-chains was normal, but comparison of the pattern from the β^X-chains with that from the β^A-chains (fig 2) showed the absence of both β^ATpIII (β18–30) which normally gives a positive staining reaction for arginine, and β^ATpIV (β31–40) which normally contains tryptophan tyrosine and arginine A new peptide was present on the fingerprint of the β^X-chain This peptide (fig 2) was negatively charged at pH 6 4, but remained at the origin during electrophoresis because electroendosmosis counteracts its migration towards the anode Its chromatographic mobility was between that of β^ATpIII and β^ATpIV and it gave positive staining reactions for tyrosine tryptophan and arginine The fingerprint of the globin from the isopropanol precipitate also showed the same abnormality

The amino acid composition of the purified variant peptide (table I) resembled that of β^ATpIII plus β^ATpIV but with one of the two arginine residues β30 and β40 (fig 3) replaced by a serine residue The most likely explanation for this finding is that arginine β30 has been replaced by serine (fig 3) which precludes the usual tryptic hydrolysis between arginine

Residue No	[illegible]	19	20	[illegible]	22	23	24	25	26	27
Helical No	A14	B1	B2	B3	B4	B5	B6	B7	B8	B9
Residues										
Hb A	↓Val	Asn	Val	Asp	Glu	Val	Gly	Gly	Glu	Ala
Hb Tacoma	↓Val	Asn	Val	Asp	Glu	Val	Gly	Gly	Glu	Ala

28	29	30	31	32	33	34	35	36	37	38	[illegible]	40
B10	B11	B12	B13	B14	B15	B16	C1	C2	C3	C4	C5	C6
Leu	Gly	Arg↓	Leu	Leu	Val	Val	Tyr	Pro	Trp	Thr	Gln	Arg↓
Leu	Gly	SER	Leu	Leu	Val	Val	Tyr	Pro	Trp	Thr	Gln	Arg↓

↓ Indicates the position of hydrolysis by trypsin

Fig 3 Amino acid sequence of the region β18–40 from Hb A and Hb Tacoma.

β30 and leucine β31 The variant is, therefore, identical with Hb Tacoma, β30 (B12) Arg→Ser [1, 3]

DEAE Sephadex chromatography resolved the haemolysate into three fractions (fig 4) in the following relative amounts Hb A_2 3 7%, Hb A 53 9% and Hb Tacoma 42 4% Oxygen affinity measurements on Hb A and Hb Tacoma, isolated under identical conditions, showed that between pH 6 0 and 7 4 the affinities of Hb A and Hb Tacoma were nearly the same (fig 5, and table II) but at pH 7.5 and 7 9 the affinity of Hb Tacoma appeared [illegible] be slightly lower than that of Hb A This may indicate a small reduction in the Bohr effect above pH 7 4, a finding which we hope to pursue in future investigations The haem-haem interaction of Hb Tacoma was normal, with an average n value of 2 9

Discussion

Arginine β30 (B12) forms part of the $\alpha 1\beta 1$ interface [14] and is in contact with the side-chains of α117 (GH5) Phe and β122 (H5) His The replacement of this arginine by serine, with its smaller side-chain, would be expected to cause a loss of these contacts and a weakening of the $\alpha 1\beta 1$ interface, favouring dissociation into monomers, which [illegible] the probable cause of the slight instability of Hb Tacoma PERUTZ [13] suggested that

Table II Oxygen affinity data for Hb A and Hb Tacoma

Hb	pH	P_{50} mmHg	$\log_{10}P_{50}$	Met Hb[1], %	
				before	after
A	6.03	13.5	1.13	4.0	12.2
Ta	6.03	15.2	1.18	7.4	12.2
A	6.2	16.0	1.20	5.2	11.6
Ta	6.2	14.4	1.16	8.0	16.0
A	6.31	17.1	1.23	1.2	5.9
Ta	6.40	15.9	1.20	5.2	9.0
A	6.81	11.8	1.07	2.4	7.1
Ta	6.82	11.7	1.07	5.0	9.6
A	7.01	10.7	1.03	1.6	ND
Ta	7.00	9.8	0.99	ND	6.6
A	7.20	7.5	0.89	2.8	5.8
Ta	7.19	8.3	0.92	6.7	13.2
A	7.40	5.9	0.77	0.0	5.4
Ta	7.40	6.0	0.78	4.0	9.6
A	7.57	3.9	0.59	2.5	6.8
Ta	7.57	5.0	0.70	6.3	8.0
A	7.86	2.6	0.41	0.0	7.8
Ta	7.85	3.9	0.59	5.5	8.0

The average n value between pH 6.2 and 7.6 = 2.9 for Hb A and Hb Tacoma, ND = not determined

[1] Determined spectrophotometrically before and after each measurement

the imidazole group of α122(H5) His may make some contribution (not more than 25%) to the Bohr effect It was reported that in the oxy-structure the imidazole group lay near the positively charged guanidinium group of β30 (B12) Arg, which would lower its pK_a, and that in the deoxy-structure it lay nearer the negatively charged side-chain of α126 (H9) Asp, which would raise its pK_a Thus, on going from the oxy- to the deoxy-structure there would be a rise in pK_a of the imidazole group, causing an uptake of protons and a contribution to the Bohr effect The replacement of the positively charged Arg by the electrically neutral Ser would be expected to reduce this effect, if it exists, and the observed difference in affinity between Hb Tacoma and Hb A above pH 7.4 suggests that there may be a slight reduction of the Bohr effect This result is being investigated more fully

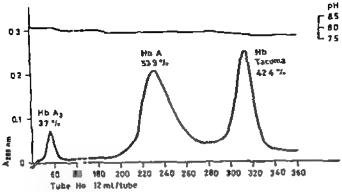

Fig 4 DEAE Sephadex chromatography [8] of baemolysate showing separation of CO Hb A from CO-Hb Tacoma

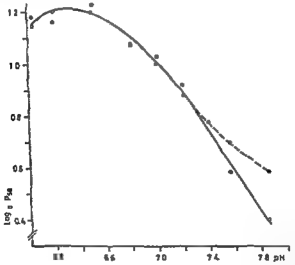

Fig 5 The variation of $\log_{10}P_{50}$ with pH for Hb A (○) and Hb Tacoma (●) P_{50} is the pressure of O_2 in mm Hg, required to attain half saturation of the haemoglobin

The substitution of arginine by serine does not appear to result in a marked change in the net charge of the Hb Tacoma tetramer, despite the overall loss of one positively charged group per β-chain Hb Tacoma barely separated from Hb A during starch gel electrophoresis at pH 8.3,

Table II Oxygen affinity data for Hb A and Hb Tacoma

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A	6.81	11.8	1.07	2.4	7.1
Ta	6.82	11.7	1.07	5.0	9.6
A	7.01	10.7	1.03	1.6	ND
Ta	7.00	9.8	0.99	ND	6.6
A	7.20	7.5	0.88	2.8	5.8
Ta	7.19	8.3	0.92	6.7	13.2
A	7.40	5.9	0.77	0.0	5.4
Ta	7.40	6.0	0.78	4.0	9.6
A	7.57	3.9	0.59	2.5	6.4
Ta	7.57	5.0	0.70	6.3	8.0
A	7.86	2.6	0.41	0.0	7.8
Ta	7.85	3.9	0.59	5.5	5.0

The average n value between pH 6.2 and 7.6 = 2.9 for Hb A and Hb Tacoma. ND = not determined.

[a] Determined spectrophotometrically before and after each measurement.

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Request reprints from Prof H Lehmann MRC Abnormal Haemoglobin Unit, University Department of Biochemistry, Addenbrooke's Hospital Hills Road, *Cambridge CB2 2QR* (England)

and did not separate at all in the other electrophoretic systems. It could, however, be separated by DEAE-Sephadex chromatography using a very shallow pH gradient (fig. 4). Exposure of the individual polypeptide chains to concentrated urea solutions, which has the effect of disrupting the tertiary structure of the globin subunits, resulted in the full expression of the charge difference between the β^A and $\beta^{[illegible]}$ chains, thus permitting their separation by ion-exchange chromatography or cellulose acetate electrophoresis. Clearly then, the charge on the side-chain of arginine [illegible] is at least partially compensated by the suppression of the charge of another residue in the intact Hb A, which in Hb Tacoma is charged. If Perutz' scheme [13] is correct, this would be α122 (H5) His.

The heat and isopropanol stability tests both indicated that Hb Tacoma was only mildly unstable, which had already been suggested by the lack of any of the clinical symptoms often associated with the presence of unstable haemoglobin (in both the patient and his son). Similarly, Baur and Motulsky [1], in their original description of a family with Hb Tacoma trait, also reported that the only clinical finding of note was a slightly raised level of Hb A_2.

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Request reprints from Prof H LEHMANN MRC Abnormal Haemoglobin Unit, University Department of Biochemistry, Addenbrooke s Hospital Hills Road, *Cambridge CB2 2QR* (England)

until the end of 1971, more than 40 cases had been identified [4] In our country, the first case was referred to by OUTERIÑO *et al* [10] MCBRIDE *et al* [9] claim that a similar condition, but without serological abnormalities, should be called CDA type IV

Ultrastructural features of CDA have received increasing attention by many authors [1, 2, 6–8, 13] and the differences between the three types have been clearly outlined In CDA type I, the changes are chiefly nuclear, including chromatin bridges connecting nuclei of dividing cells, uneven condensation of nuclear chromatin with spongy appearance, widening of nuclear membrane pores and partial loss of nuclear envelope with penetration into the nuclear space of cytoplasmic components (ribosomes and haemoglobin) On the contrary, in CDA type II the ultrastructural features are chiefly cytoplasmic, consisting in the formation of a complete or discontinuous 'double cytoplasmic membrane' in most nucleated cell precursors and in some erythrocytes There is only one known case of CDA type III with ultrastructural study [2] Here, again, the changes are predominantly nuclear, similar to those found in CDA type I However, the presence of gigantoblasts, and earlier and more striking multinuclearity seems to permit the separation from CDA type I, where the alterations are less pronounced and begin from more mature stages of red cell precursors

This report includes a clinical and ultrastructural study of a case of CDA type II In addition to the 'double cytoplasmic membranes' typical of CDA type II, some nuclear alterations similar to those described in CDA type I were found

Case Report

M R This young woman was born in 1939 following a normal full term pregnancy but showed a low birth weight (1,500 g) A family history of anaemia was lacking Though developing normally the patient was always easily fatigued At the age of 12 years a splenomegaly was apparently felt In 1957, the diagnosis of hepa[illegible]

In 1967 she was believed to have a congenital haemolytic anaemia Her liver was felt 5 cm below the right costal margin and her spleen 10 cm below the left costal margin The most outstanding features at that time were a medium degree anaemia with normocytic peripheral blood picture a slight erythroblastaemia (2%) and a normal or low reticulocyte count The sternal bone marrow examination yielded

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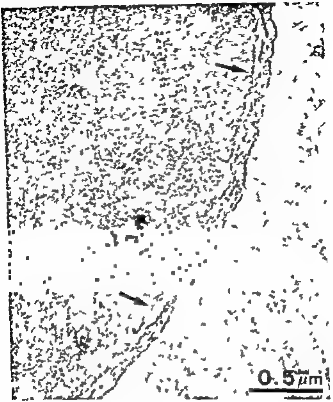
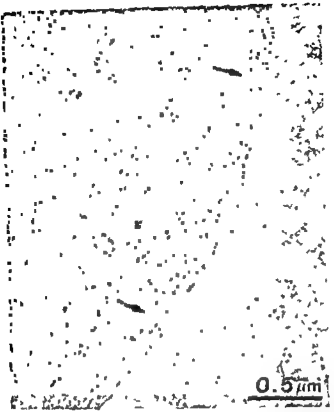


Fig 2 Cytoplasmic double membrane appearance (arrows) in late erythroblast × 40 000

The sternal bone marrow was markedly hypercellular with 65% of red precursors Less than 5% of early erythroblasts and less than 20% of intermediate erythroblasts had 2 nuclei 39% of late erythroblasts had more than one nucleus (32% two and 9% more than two nuclei) (fig 1) There were 70% of sideroblasts and reticulum cell iron was increased 4% of red cell precursors showed fine granular PAS positivity

Electron microscopy The following method was used aspirated sternal marrow was placed directly into cold 2% glutaraldehyde in 0 1 M phosphate buffer Marrow particles were then separated and fixed in fresh 2% glutaraldehyde for 2 h at 4 °C, rinsed in 0 1 M phosphate buffer with sucrose post fixed in osmium tetroxide dehydrated in increasing series of acetone and embedded in Vestopal W The sections were cut on Reichert s ultramicrotome mounted on carbon-coated copper



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grids, stained with uranyl acetate and lead citrate and examined in a JEOL 100-B electron microscope at 60 kV

Ultrastructural findings Proerythroblasts appeared normal Excessive intracellular membranes looking like double cytoplasmic membranes (fig 2) were seen in more than 65% erythroblasts of all stages of maturation, but were more striking in late erythroblasts In some cells, predominantly in those with two or more nuclei, the 'double membranes were seen uninterruptedly along the whole cell perimeter, but usually they were discontinuous Rhopheocytosis seemed to be impaired at the sites of 'double membrane', but remained undisturbed at other points There was increased iron in most cytoplasms, sometimes in the mitochondria In late erythroblasts, polyribosomes appeared to be less frequent than normal In addition to cytoplasmic changes, nuclear abnormalities were observed These included uneven condensation of chromatin, which was denser than normal Contrary to normal erythroblasts, the non-condensed chromatin (euchromatin) contacted the nuclear envelope much more extensively (fig 3, 4) However, the nuclear envelope did not disappear nor did penetration of cytoplasmic components into the nuclear region occur These nuclear changes were less frequent (some 50% of erythroblasts) than the cytoplasmic ones Usually both were coincidental, but some cells exhibited only cytoplasmic disturbances, while more rarely there were only nuclear alterations Red cell and red cell precursors phagocytosis by reticulum cells was observed In addition, many inclusions, myelin figures and iron particles were seen in these cells In 35% of the red cells, reticulocytes were seen to included 'double membrane forma tion, which was, however, usually discontinuous Furthermore, increased iron content was noted in erythrocytes

Family studies The complete blood count and red cell morphology were normal in the patient s mother and in her three children In addition, the red cell precursors were normal by light and electron microscopy in the children

Discussion

The most interesting findings of the case are at ultrastructural level The presence of the cytoplasmic 'double membrane' is typical of CDA type II and further confirms the diagnosis established by other criteria But we would like to emphasize the nuclear changes found in our patient Normal erythroblasts show condensed chromatin (heterochromatin) all around the nuclear periphery interrupted only at the narrow areas of nuclear pores where non condensed chromatin (euchromatin) contacts the inner face of the nuclear membrane In our case, there is extensive con-

Fig 4 Central part (square area) of figure 3 at higher magnification The nuclear membrane does not disappear in the areas of contact with euchromatin. × 30 000

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Request reprint from Prof C ROZMAN, Hospital Clinico y Provincial, Casanova 143, *Barcelona 11* (Spain)

Progress in Fibrinolysis

Postgraduate course and workshop organized by the European Thrombosis Research Organization and the Fondazione Giovanni Lorenzini. Milan, March 6-8, 1975

Scientific committee: M. B. Donati, P. M. Mannucci, V. Marder, I. M. Nilsson and M. Verstraete

This three-day course, held in English and directed to research workers in the field, deals with recent advances in the theoretical and clinical problems related to fibrinolysis. The morning sessions, opened to a maximum of 300 applicants, will include lectures given by invited speakers followed by open panel discussion and question periods which will attempt free dialogue with the audience. The afternoon workshop opened to 100 applicants, selected on the basis of their curriculum vitae showing a specific interest in the field, will be based on demonstration and discussion of laboratory methods including the recent advances in the field.

Topics: The fibrinolytic system and haemostasis. Relationship between fibrinolysis and other biological systems. Biochemistry of plasminogen. Mechanisms of plasminogen activation. Tissue localization of plasminogen activator. Plasmic degradation of fibrinogen. Differentiation of fibrinogen and fibrin degradation products. Non plasmin proteolysis of fibrinogen and fibrin. Plasmin degradation of factor VIII related material. Clinical significance of fibrinolysis. Local fibrinolysis as a mechanism for haemorrhage. Indications for antifibrinolytic drugs. Activation of the fibrinolytic system with non thrombolytic agents. Clinical value of urinary FDP. Round table discussion on thrombolytic therapy.

Workshop discussions of the following methods: Euglobulin lysis time. Fibrin plates. Plasminogen assay. Inhibitors of fibrinolysis. Todd's methods. Fibrin lysis in cultured cells. Survival of radiolabelled proteins. Fibrinogen degradation products. Radioimmunoassay for differentiation between fibrinogen and fibrin degradation products. Radioimmunoassay fibrinopeptide A.

All registrations and requests for information are to be directed before January 31st, 1975 to: Fondazione Giovanni Lorenzini, Via G. Lorenzini, 2 (ex Via Brembo 65), 20139 Milano (Italy).

Acta Haemat 52 321–330 (1974)

Myoglobin in Homozygous β-Thalassaemia [1]

BASIL ANGELOPOULOS, KOSTAS VLASSOPOULOS and ALEX KALOS

Department of Pathologic Physiology, Sections of Internal Medicine and Research Laboratory, National University of Athens, (Director Prof B ANGELOPOULOS) Athens

Key Words
Haemoglobin
Mediterranean anaemia
Myoglobin
Thalassaemia

Abstract Purified, haemoglobin free oxymyoglobin preparations obtained from heart tissue of adult subjects (Mb A) were compared with myoglobin obtained from heart tissue of fetuses (Mb F) and of individuals with homozygous β thalassaemia (Mb C) It was found that myoglobin from individuals suffering from homozygous β thalassaemia was different from that of fetuses and adults Electrophoretic studies, particularly on acrylamide gel and on cellulose acetate, showed characteristic differences of the components Two-dimensional peptide maps (fingerprints) showed the presence of a new peptide in Mb C, which was not found in Mb A and Mb F It appears that the presence of this peptide is associated with a characteristic change in the structure of the Mb C molecule

It is well known that there are 3 forms of haemoglobin, the fetal (Hb F) found in the fetus and the newborn and those present in normal adults (Hb A and Hb A_2) In the homozygous β thalassaemia (Mediterranean anaemia) the presence of fetal haemoglobin in the adult indicates that its replacement adult form is likewise governed by hereditary factors There is a paucity of information in the literature with respect to the appearance of most of the other proteins and particularly of myoglobin in patients suffering from Mediterranean anaemia Different diseases in which myoglobins are expected to be disturbed have been studied [5, 23, 26 38] Very limited information is available on myoglobin in hereditary haemolytic anaemia and this is mainly confined to sickle cell anaemia [20, 28, 32, 33]

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Workshop discussions of the following methods Euglobulin lysis time Fibrin plates Plasminogen assay Inhibitors of fibrinolysis Todd s methods Fibrinolysis in cultured cells Survival of radio labelled proteins Fibrinogen degradation products Radioimmunoassay *for differentiation between fibrinogen and fibrin degradation* products Radioimmunoassay fibrinopeptide A

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In the present study, the physical and chemical properties of myoglobin in individuals suffering from homozygous β-thalassaemia was examined and compared with myoglobin of fetuses and adults.

Material and Methods

Heart muscle tissues were obtained during post mortem examination from 10 adults, who died in traffic accidents, 10 fetuses 24–28 weeks of gestation and 3 children 7, 8 and 13 years old, who died from homozygous β thalassaemia.

Tissue samples were obtained 4 h after death and were immediately stored at −20 °C until use. After removal of fat and ligaments, the heart muscle was shredded, then 1 ml of distilled water per gram of muscle tissue was added and the specimen obtained was homogenized in an electric homogenizer at 0 °C and spun at 25 000 rpm for 30 min at −10 °C, to remove the particulate matter. The supernatant liquid was concentrated employing one of the following methods: (1) Lyophilization using the VirTis freeze drying apparatus (from Ocean Side), (2) concentration by absorption on Lyphocel (polyacrylamide hydrogel from Gelman), and (3) by 24-hour dialysis through ¾ cellulose-tubing from the Visking Co., against large volumes of distilled water at 4 °C.

To prepare a pure myoglobin dilution, the material was dissolved, after lyophilization, in 0.2–0.5 ml of 0.5 M sodium phosphate buffer solution (pH 7.4). If it was concentrated, the same solution was used. In both instances the density of the samples was raised by adding sucrose 25% (w/v). Freeze-drying produced a more concentrated myoglobin sample. Myoglobin was separated from the haemoglobin by chromatography [15].

Normal human haemoglobin was prepared by lysis of freshly washed red cells obtained from the blood of healthy adults (Hb A) and cord blood from newborns (Hb F).

To avoid complications that may arise from autoxidation of myoglobins and to eliminate any heterogeneity that disappears in the presence of cyanide, the myoglobins and haemoglobins were [illegible] to cyan met form [25].

The myoglobins (Mb A, Mb F and Mb C) were analyzed electrophoretically and compared with the haemoglobins (Hb A and Hb F) by means of: (1) spectroscopy: ultraviolet [11] and visible [35] spectra (260–700 nm) were recorded in a Beckman DU 2 spectrophotometer using 1.0-cm quartz cells; (2) production of pure crystalline myoglobin using Lugnauer's [22] method; (3) resistance of myoglobin to alkali according to Jonxis and Wadman's [20] method; (4) determination of the iron content of myoglobin according to the method of Dietrich and Schmitt [13] and Gubler *et al* [17]; (5) electrophoresis on various media and at different pH: on paper at pH 8.8 in Tris buffer [12], cellulose acetate [16], starch gel in alkaline and acid buffer and hydrolyzed starch (Connaught, Toronto) [34] and acrylamide gel [24]; (6) digestion of myoglobin with trypsin and chymotrypsin according to the method of Hill *et al* [18]; and (7) two-dimentional electrophoresis-chromatography peptide analysis "fingerprinting" according to the method of Ingram [19] as modified by Baglioni [2]. The color re-

actions specific for various amino acids were carried out on duplicate fingerprints of each subject [6]

Results

Chromatography During chromatographic separation on a Sephadex column, it was noted that the size of the haemoglobin band was inversely proportional to its concentration in the muscle preparation It was also observed that haemoglobin migrates at a more rapid rate than does myoglobin

Spectroscopy We observed no demonstrable differences between fetal, adult and β thalassaemia myoglobin, when measuring the absorption spectra in visible and ultraviolet light Our findings are in keeping with those in the literature [4, 14, 32, 35, 36]

Crystallization In the oxy- and meta forms of myoglobins we observed needle shaped, doubly refractive crystals of approximately 1 mm diameter, with no visible differences between each type of myoglobin

Resistance to alkali As is well known, myoglobin is very resistant to alkali At room temperature it is not denatured No important difference in the production rate of the alkaline haemochromogen was found between fetal and adult myoglobin, while the rate of reaction at 46 °C for

Table 1 Iron content of myoglobins (%)

	Mb A n = 10	Mb F n = 10	Mb C n = 3
	0 288	0 365	0 402
	0 278	0 339	0 408
	0 265	0 330	0 396
	0.299	0 342	
	0 302	0 362	
	0 286	0.352	
	0 296	0 336	
	0 303	0 350	
	0 306	0 333	
	0 297	0 341	
Mean	0.292	0.345	0 402

n = Number of cases

In the present study, the physical and chemical properties of myoglobin in individuals suffering from homozygous β thalassaemia was examined and compared with myoglobin of fetuses and adults.

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Heart muscle tissues were obtained during post mortem examination from 10 adults who died in traffic accidents, 10 fetuses 24–28 weeks of gestation and 3 children 7, 9 and 13 years old who died from homozygous β thalassaemia.

Tissue samples were obtained 4 h after death and were immediately stored at −20 °C until use. After removal of fat and ligaments, the heart muscle was sliced, then 1 ml of distilled water per gram of muscle tissue was added and the specimen obtained was homogenized in an electric homogenizer at 0 °C and spun at 25 000 rpm for 30 min at −10 °C, to remove the particulate matter. The supernatant liquid was concentrated employing one of the following methods: (1) Lyophilization using the VirTis freeze drying apparatus (from Ocean Side); (2) concentration by absorption on Lyphogel (polyacrylamide hydrogel from Gelman), and (3) by 24-hour dialysis through a cellulose-tubing from the Visking Co. against large volumes of distilled water at 4 °C.

To prepare a pure myoglobin dilution, the material was dissolved after lyophilization in 0.2–0.5 ml of 0.5 M sodium phosphate buffer solution (pH 7.4). If it was concentrated, the same solution was used. In both instances the density of the sample was raised by adding sucrose 25% (w/v). Freeze-drying produced a more concentrated myoglobin sample. Myoglobin was separated from the haemoglobin by chromatography [15].

Normal human haemoglobin was prepared by lysis of fresly washed red cells obtained from the blood of healthy adults (Hb A) and cord blood from newborns (Hb F).

To avoid complications that may arise from autoxidation of myoglobins and to eliminate any heterogeneity that disappears in the presence of cyanide, the myoglobins and haemoglobins were analyzed electrophoretically and to cyan met form [25].

The myoglobins (Mb A, Mb F and Mb C) were compared with the haemoglobins (Hb A and Hb F) by means of: (1) spectroscopy: ultraviolet [11] and visible [35] spectra (260–700 nm) were recorded in a Beckman DU 2 spectrophotometer using 1.0-cm quartz cells; (2) production of pure crystalline myoglobin using LUGINBUHL's [22] method; (3) resistance of myoglobin to alkali according to JONXIS and WADMAN's [20] method; (4) determination of the iron content of myoglobin according to the method of DIETRICH and SCHMITT [13] and GUBLER *et al.* [17]; (5) electrophoresis on various media and at different pH: on paper at pH 8.8 in Tris buffer [12], cellulose acetate [16], starch gel in alkaline and acid buffer and hydrolyzed starch (Connaught, Toronto) [34] and acrylamide gel [24]; (6) digestion of myoglobin with trypsin and chymotrypsin according to the method of HILL *et al.* [15] and (7) two-dimentional electrophoresis-chromatography, peptide analysis, fingerprinting, according to the method of INGRAM [19] as modified by BAGLIONI [2]. The color re-

actions specific for various amino acids were carried out on duplicate fingerprints of each subject [6]

Results

Chromatography During chromatographic separation on a Sephadex column, it was noted that the size of the haemoglobin band was inversely proportional to its concentration in the muscle preparation It was also observed that haemoglobin migrates at a more rapid rate than does myoglobin

Spectroscopy We observed no demonstrable differences between fetal, adult and β-thalassaemia myoglobin, when measuring the absorption spectra in visible and ultraviolet light Our findings are in keeping with those in the literature [4, 14, 32, 35, 36]

Crystallization In the oxy- and meta forms of myoglobins we observed needle-shaped, doubly refractive crystals of approximately 1 mm diameter, with no visible differences between each type of myoglobin

Resistance to alkali As is well known, myoglobin is very resistant to alkali At room temperature it is not denatured No important difference in the production rate of the alkaline haemochromogen was found between fetal and adult myoglobin, while the rate of reaction at 46 °C for

Table I Iron content of myoglobins (%)

	Mb A $n=10$	Mb F $n=10$	Mb C $n=3$
	0.288	0 365	0 402
	0 278	0 339	0 408
	0 265	0 330	0 396
	0 299	0 342	
	0 302	0 362	
	0.286	0.352	
	0 296	0 336	
	0 303	0 350	
	0 306	0 333	
	0 297	0 341	
Mean	*0.292*	*0.345*	*0 402*

n = Number of cases

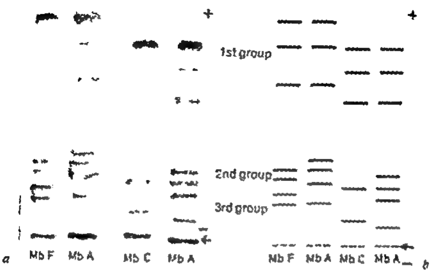


Fig 1 Myoglobins Mb A Mb F and Mb C at pH 8 9 *a* Electrophoresis on cellulose acetate *b* Tracing of electrophoretic patterns, ← = origin

Mb C was clearly quicker (180 min) than that of Mb A and Mb F (200–210 min)

Iron content The iron content of myoglobin is shown in table 1 It is suggested that the increase in iron content in the myoglobin of heart muscle in patients suffering from β-thalassaemia was due to haemochromatosis resulting from blood transfusions

Electrophoresis On paper, no differences were found between the 3 myoglobins On cellulose acetate 3 distinctly different groups were observed (fig 1) The first group moved the fastest towards the anode and was composed of 3 components which were found to be identical in Mb A, Mb F and Mb C The second group moved more slowly and consisted of 3 components in Mb A, 2 components in Mb F and 1 component in Mb C Finally, the third group which was the slowest, was found near the cathode and was composed of 1 component in Mb A 2 components in Mb F and 1 component in Mb C On starch gel using an alkaline buffer solution of pH 8 8, we observed 4 components in Mb A and Mb F and an additional, more slowly moving component, in Mb C In a buffer solution of pH 6 0 Mb A and Mb F had 3 components while Mb C showed an additional, more slowly moving component On acidic starch gel haemoglobin moved slowly and the various forms did not separate from each other On acrylamide (12 g acrylamide 100 ml gel, fig 2) we observed 3

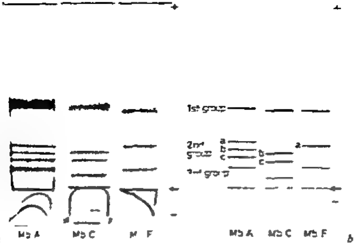
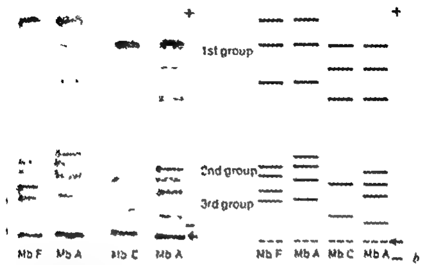


Fig. 2. Myoglobins Mb A, Mb F and Mb C at pH 9.5. *a* Electrophoresis in acrylamide gel. *b* Tracing of electrophoretic patterns. ← = origin.

groups of components. The first consisted of 1 component which moved fastest toward the anode and was very distinct in Mb A and Mb C and barely discernible in Mb F. The second group consisted of 3 components (a, b and c) in Mb A, of 1 component (a) in Mb F and 2 components (b and c) in Mb C. Finally, the third group migrated close to the cathode and consisted of 1 component which moved at the same rate for Mb A and Mb F and more slowly in Mb C. In cyan-met forms, the electrophoretical findings were similar.

Tryptic and chymotryptic digestion of globin. The rates of acid hydrolysis for Mb A, Mb F and Mb C did not differ.

Fingerprinting. Using paper electrophoresis followed by ascenting chromatography, two-dimensional peptide maps (fingerprints) were prepared. The results are illustrated in figure 3. The numbering is according to INGRAM's [19] classification. Upon comparison of the fingerprints of Mb A, Mb F and Mb C, we found the same peptides, except for peptide No. 6, which was found only in Mb A. In Mb C, we found an extra peptide to which we assigned No. 42. This peptide was rendered visible with ninhydrin and was not found in Mb A or Mb F. Attempts to determine the amino acids of this new peptide using chymotryptic digestion were unsuccessful. The fact that we succeeded in finding this new peptide in 3



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terpart Moreover, ROSSI FANELLI *et al* [30] found that the amino acid composition of myoglobin was similar in adults and infants BENOIT *et al* [3] described the presence of fetal myoglobin in the urine of an adult with idiopathic myoglobinuria

PERKOFF *et al* [25] reported at least 4 distinct haemoproteic components chromatographically on DEAE-cellulose The 2 chromatographic components that account for 75–80% of total myoglobin of normal human muscle appear to differ from each other in their prosthetic haem groups BOYER *et al* [10] studied ultrafiltrates of human skeletal muscle by starch-gel electrophoresis and found 2 variants, named Mb Aberdeen and Mb Annapolis Neither variant was fully characterized Combined electrophoresis and chromatography of Mb Annapolis indicated a loss of arginine at the C terminal position of the molecule BOULTON *et al* [8, 9], in a survey of 6,000 human muscle samples, found 4 myoglobin variants by paper electrophoresis It has been possible to identify 2 of the 4 components 53(D_4) Glu→Lys [7] and 138(H_{14}) Arg→Glu, which had a particular interest because it may be identical with the above mentioned Mb Annapolis

In the present investigation, characteristic differences between Mb A, Mb F and Mb C were shown in particular in the migration rates of components, especially on cellulose acetate and acrylamide gel electrophoresis as well as in the two-dimentional peptide maps Such differences usually denote differences in the structure of the protein molecule

The peptides shown in fingerprints from Mb A, Mb F and Mb C were identical, with the exeption of No 6, which was found only in Mb A In Mb C, we found a new peptide to which we gave the number 4a It is possible that this peptide was identical with the one described by BOYER *et al* [10], but we could not find the characteristic components of Mb Annapolis on starch-gel electrophoresis of Mb C

Since human myoglobins contain -Lys Lys bonds in positions 61, 62 and -Lys-Lys-Lys bonds in positions 76–78, it is impossible to digest completely these bonds by trypsin as shown in the paper of HILL *et al* [18] on the sequence determination of human myoglobin As frequently experienced in the peptide mapping of haemoglobin chains, the relative intensities of the peptide spots, produced by incomplete proteolysis are different from experiment to experiment and it is practically impossible to control this difference Therefore we believe that the extra peptide spot (4a) found in the myoglobin from a thalassaemic subject could neither be explained by such ambiguous proteolysis as haemoglobin nor as second-

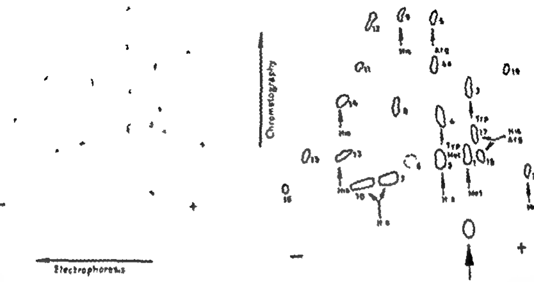

Fig 3 Two-dimentional electrophoresis-chromatography of tryptic digest of heart myoglobin from a patient with homozygous *β* thalassaemia *a* Fingerprint *b* Tracing, showing the system for numbering peptide following INGRAM's [19] classification Peptide No 6 does not occur in Mb C. Peptide No 4a was the extra peptide found, ▲ = origin

successive samples of Mb C leads us to consider it as a characteristic change in the structure of Mb C protein molecule

Discussion

Information with respect to the fetal type of muscle pigment is meagre The existence of this protein has been postulated by several investigators, drawing an analogy to the presence of a fetal haemoglobin [3, 20, 21, 27, 29, 31–33] JONXIS and WADMAN [20] indicated that human myoglobin exists in the fetal as well as adult form, in a fashion similar to that of human haemoglobin In 1955, SINGER *et al* [32, 33] demonstrated the presence of a fetal form of myoglobin in newborn infants, which has an electrophoretic mobility different from adult myoglobin Furthermore KASSMAN *et al* [21] observed a quantitative identity between adult and fetal myoglobin and their findings were in accordance with the earlier observations by SCHNEIDERMAN [31] who found no difference between the fingerprints of myoglobin derived from the muscle of infants and its adult coun-

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ary artifacts of post-mortem denaturation of protein molecule, since we could not find this spot (4a) in any of the numerous experiments in tissues from fetuses and adult subjects We therefore believe that Mb C differs both from Mb A and Mb F

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Acta Haemat. 52 331–335 (1974)

High Transfusion Regime in the Management of Reproductive Wastage and Maternal Complications of Pregnancy in Thalassaemia major

A M AFIFI

Abstract Eighteen Egyptian females with β thalassaemia major were closely followed up over a 6-year period for the evaluation of their pregnancy outcome under 2 different types of transfusion regime In the first 3 years during which the haemoglobin level of all the gravidas was maintained by minimal transfusions at a mean value of 6 6 g/100 ml the over all reproductive wastage was 34.5% and 4 mothers developed complications When maternal haemoglobin was maintained during the following 3-year period at a level above 10 g/100 ml the overall reproductive wastage dropped to 13 6% and virtually no maternal complications developed The high transfusion regime used to maintain this high maternal haemoglobin level was well tolerated and except for 1 case of serum hepatitis, no serious side effects were recorded

Key Words
Haemoglobinopathies
Pregnancy in thalassaemia
Thalassaemia major
Transfusions in thalassaemia

Sucessful pregnancy in females suffering from β thalassaemia major with a haemoglobin level below 7 3 g/100 ml is uncommon Although this is partly due to the small number of females with thalassaemia major who survive beyond the second decade, other factors responsible for this finding are the retarded physical and sexual development of the survivors with poor chances for marriage, the frequent development of maternal complications recommending pregnancy interruption, and finally the large number of pregnancies that end as reproductive wastage

The maternal hazards in β thalassaemia major are cardiac failure susceptibility to infections inability to withstand important blood loss and leg ulcers Excluding the latter condition which is peculiar to thalassaemia and haemoglobinopathies all the others may develop in any severely anaemic gravida [1] The reproductive wastage is due to the high rates of

spontaneous abortion, prematurity and stillbirth. These conditions are responsible for the fetal wastage in severe anaemia in general, whether due to haemoglobinopathies [1] or other causes [3, 4].

Since the survival of patients with β-thalassaemia major is directly dependent on supportive treatment by blood transfusion and folic acid, energetic management of intercurrent illness and the judicious use of splenectomy, the availability of thalassaemia centres providing medical care along these lines has resulted in the survival of a growing number of increasingly old patients. Although these patients manifest satisfactory physical and sexual development with better chances for marriage, they still suffer virtually the same rate of reproductive wastage. This implies that the improved survival and health of females with β-thalassaemia major will increase the magnitude of the problem of fetal wastage.

The role of the high transfusion regime in preventing the development of complications in patients with β-thalassaemia major has been evaluated by WOLMAN [5]. He reported, in addition to enhancement of growth, significant reduction in the incidence rate of cardiac enlargement, bone malformations and hepatosplenomegaly. These encouraging results stimulated us to study the value of high transfusion regime in reducing the rate of maternal and fetal complications in thalassaemic gravidas. This paper reports our results in maintaining the maternal haemoglobin level above 10 g/100 ml in pregnant females with β-thalassaemia major who suffered both high rates of reproductive wastage and maternal morbidity in previous pregnancies.

Material and Methods

The 18 female patients in this trial were all patients of our thalassaemia clinic for whom haemoglobin and haematocrit determinations as well as alkali denaturation test and starch gel haemoglobin electrophoresis were performed. The average age of the patients was 26 years, the youngest being 23 and the oldest 36. The haemoglobin levels of the 18 patients, who were all of high social class and receiving proper medical care, ranged from 6.2 to 7.1 g/100 ml, with fetal haemoglobin levels between 16 and 38%.

The patients were followed dur[illegible] ears of their marital life starting from the
date of their first conception. In [illegible] ars the 18 patients had 29 pregnancies
during which maternal haemoglo[illegible] ined at the usual level that keeps
the gravida free of symptoms of [illegible] er lines of treatment includ[illegible]
prevention or early management of [illegible] ance of adequate levels [illegible]
and energetic management of [illegible] properly carried out

ly haematocrit and haemoglobin determinations and occasional reticulocyte count were also performed In the second 3-year period they had 22 pregnancies during which maternal haemoglobin was maintained by high transfusion regime above 10 g/100 ml starting from the 8th week of pregnancy to term The supportive measures and the haematologic check ups were carried out in the above mentioned fashion

Results

Apart from minor transfusion reactions the high transfusion regime was well tolerated by all the patients in this trial No signs of circulatory overload were encountered provided a maximum of 2 units of packed red cells twice weekly was not exceeded and the patient kept in the sitting position during transfusion Only 1 patient developed serum hepatitis 12 weeks after a full term pregnancy

Table I demonstrates that in the first 3 year period of the study during which only safe maternal haemoglobin level was maintained, 10 (34 5%) out of 29 conceptions ended as reproductive wastage Although there were only 8 fetal losses, 6 spontaneous abortions and 2 stillbirths, 2 of the 21 live born infants died in the meonatal period because of prematurity This raised the overall reproductive wastage from 8 to 10 Seven (33 3%) of the 21 live born infants (birth weights ranging from 1,200 to 1 850 g) were prematures and as mentioned before, prematurity was re-

Table I Reproductive wastage under the transfusion regimes

Forms of reproductive wastage	Maternal haemoglobin g/100 ml	
	less than 7 3	above 10
Total number of pregnancies	29	22
Maternal mortality	0	0
Maternal morbidity		
Heart failure	2	0
Leg ulcers	2	1
Abortions	6	2
Low birth weight (less than 2,500 g)	7	3
Stillbirth	2	1
Neonatal death	2	0
Total reproductive wastage	10 (34 5%)	3 (13 6%)

sponsible for 2 neonatal deaths There was no maternal mortality, but 2 mothers developed cardiac failure and another 2 developed bilateral perimalleolar leg ulcers These figures contrast with those obtained in the succeeding 3-year period, during which maternal haemoglobin was maintained over 10 g/100 ml, where only 3 (13 6%) out of 22 pregnancies ended as reproductive wastage, 2 as spontaneous abortion and 1 as stillbirth Mild degree of prematurity with a birth weight between 1,820 and 2,300 g was noted for 3 live-born infants none of whom died in the neonatal period Apart from 2 active leg ulcers in 1 patient, no maternal complications were recorded

Discussion

The successful supportive measures responsible for the survival of the patients in this trial to the reproductive age were continued without modification in the first 3-year period after their marriage A haemoglobin level with a mean value of 6 6 g/100 ml to which the patients had been safely and comfortably adjusted was also maintained during all pregnancies in this period Apart from the maintenance requirements needed to keep maternal haemoglobin at this level, transfusions were given only in emergency situations as preparing patients for obstetric surgical produces, treatment of aplastic crises, or management of acute blood loss The pregnancy outcome during this period revealed an over-all reproductive wastage of 34 5% and a low birth weight incidence of 33 3% of the live-born infants

Such fetal loss is especially depressing for the thalassaemic gravida since each pregnancy represents a risk to her life or at least a period of stress during which complications develop with high frequency The former figures contrast with a reproductive wastage of 13 6% and a low birth weight incidence of 15% of the live-born infants recorded in the second 3-year period after marriage during which maternal haemoglobin was maintained at a level above 10 g/100 ml This marked drop in reproductive wastage and low birth weight incidence provides statistically significant evidence ($P<0\ 01$) supporting the value of using high transfusion regime to keep the haemoglobin level in thalassaemic gravidas above 10 g/100 ml Except for leg ulcers in 1 patient the high transfusion regime prevented the development of maternal complications

Although there has been considerable controversy regarding the real

benifits of high transfusion regime as a long term supportive measure in thalassaemia, its selective use in pregnancy seems more convincing Since recent studies suggest that intrauterine hypoxia in the severely anaemic gravidas leads to prematurity, pre labour intrauterine death and *intra partum* death, the maintenance of a high maternal haemoglobin level prevents all these hazards by providing adequate placental perfusion and oxygenation The number of transfusions required to maintain maternal haemoglobin level above 10 g/100 ml during an average of 2 pregnancies is too small to cause exacerbation of visceral siderosis, a major side effect of long term use of the high transfusion regime Screening the blood bottles for Australia antigen, which was not done in this trial would protect the patients against serum hepatitis, another major hazard of the high transfusion regime

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Acta Haemat. 52 336-344 (1974)

Increased Capillary Permeability in Haemophilia and Afibrinogenaemia

Possible Involvement of Anti-Haemophilic Factors and Fibrinogen in the Functions of the Vascular Wall

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Abstract In patients with various disorders of haemostasis and in 13 controls we studied the capillary permeability to human ^{99}Tc-albumin In 16 haemophiliacs, in 2 afibrinogenaemics and in 1 out of 3 patients with von Willebrand's disease with a factor VIII level of about 15% we observed a marked increase of capillary permeability On the contrary, 2 patients with a congenital lack of factor V, 2 cases of von Willebrand's disease with a factor VIII level of about 50% and 3 subjects with ITP showed a normal capillary permeability. The administration of a single small dose of factor VIII (1 U/kg) or IX (2 U/kg) or fibrinogen (15 mg/kg) which did not improve the clotting disorder corrected the permeability for at least 3 days The induction of a severe clotting disorder by heparin administration in man and by anticoagulant treatment in rabbits did not change the capillary permeability These results support the hypothesis that antihaemophilic factors and fibrinogen could be involved in the composition and functions of the vascular wall

Key Words
Afibrinogenaemia
Anticoagulants
Capillary permeability
Coagulation disorders
Factor VIII
Factor IX
Fibrinogen
Haemophilia

It is well established that spontaneous haemorrhages occur most frequently in haemophiliacs with a very low level of factor VIII However, there is no close relationship between the severity of the coagulopathy and the severity of the haemorrhages The bleeding, on the other hand, necessarily implies an anatomic or functional loss of integrity of the vascular wall At present very little is known regarding the way in which this may be caused by haemophiliac coagulopathy WITTE and BRESSEL [22] found that cantharidin blisters in haemophiliacs contained a greater quantity of coagulation proteins than those in controls However, this ob-

servation may only indicate a greater response of haemophiliacs to the inflammatory stimulus of cantharidin

The purpose of this work is to investigate the functional aspects of the vascular walls of the haemophiliacs compared with those of controls

Materials and Methods

Study of Capillary Permeability in Patients

Study of permeability in haemophilia We examined 16 haemophiliacs (12 A and 4 B) without inhibitors in a non haemorrhagic period 2 afibrinogenaemics and 2 subjects with congenital lack of factor V (plasmat... activity below 1%) We examined also 3 subjects with idiopathic thrombocyto... purpura (ITP) and 3 subjects affected by von Willebrand's disease two ... had a factor VIII activity of about 50% and one had an activity of ... s of von Willebrand's disease was based on a delayed increase in ... after cryoprecipitate administration and afibrinogenaemia was con... ological assay of fibrinogen (radial immunodiffusion and immune... and coagulation tests All haemophi... liacs had a factor VIII or IX le...

In all subjects with haem... we performed joint scintigrams in order to rule out any localize... rease in sites of previous haemar... throsis and we excluded 4 ha... and 1 B) with positive joint scinti... grams As controls we studied ... hy males from 15 to 25 years of age

In 11 haemophiliacs (9A a... afibrinogenaemics we evaluated the capillary permeability before a... on of factor VIII IX and fibrinogen For the administration ... commercial cryoprecipitates (Immuno Wien) 1 and 3 U/kg in a s... istration of factor IX we used a commercial preparation (Beh... gle dose of 2 U/kg. For fibrinogen we used fibrinogen Kabi (I... en 90%)

Coagulation tests In all su... the bleeding time according to Ivy *et al* [10] and carried ou... ... (Hel... ge apparatus) on whole blood Partial thromboplastin t... ... ed by the method of LANGDELL *et al* [11] using activated ... ... One-stage prothrombin time was measured by the method ... ... m thromboplastin (Behringwerke), and prothrombin consu... ... ed according to BIGGS [3] Euglobulin lysis time was perf... ... ... strom [14] and plasminogen assay by radial immunod... ... ... et al [12]. Fibrinogen assay was performed according ... ... For haemophiliacs we also carried out the thrombopl... ... g to BIGGS and DOUGLAS [4] and the assay of factor VIII ... by the ... ... EGBERG [7]. For the two subjects lacking factor V we p... ... factor V determining prothrombin time using deficient ... ... Behringwe... ... ng to SCHULTZE and SCHWICK [20]

Effect of heparin administration ... we studied capillary permeability ... ... 150 mg every 3 h

for 3 days). The anticoagulation was evaluated by PTT. Capillary permeability was measured at 8 a.m. of the 4th day. We contemporaneously collected urines for the evaluation of microhaematuria. This was measured by labelling autologous red blood cells according to MOLLISON and VEALL [15] and by assessing urinary radioactivity (cpm/urinary volume of 24 h) during 24 h for 3 days before treatment and for 3 days of heparin treatment. We began the assessment of urinary radioactivity on the 3rd day after administration of labelled red blood cells in order to rule out any possible increase of urinary radioactivity caused by red blood cell damage during labelling processes. In order to obtain a correct evaluation of the haematuria we related urinary radioactivity for each subject to the radioactivity of 1 ml packed red blood cells. All subjects were kept in bed during the test. The urines of 24 h were collected at 8 a.m. beginning on the second day of the experiment.

Study of Capillary Permeability in Rabbits

We assessed the capillary permeability for radioalbumin in three groups of rabbits: controls (5 animals), under dicoumarol treatment (5 animals) and under heparin treatment (7 animals). Dicoumarol (Coumadin®) was administered by a gastric catheter in a dosage sufficient to maintain prothrombin activity at about 10%. Capillary permeability was determined on the 5th day on prothrombin activity at about 10%. Heparin was administered by continuous infusion (peristaltic pump Sigmamotor) at dosages of 5 mg/kg/h and the capillary permeability was evaluated 30–32 h after the beginning of heparin infusion. Two animals died during heparin administration. The anti-coagulation was monitored by serial PTT.

Experimental Procedure

In patients we evaluated the capillary permeability according to MARKS and SHUSTER [13] using human radioalbumin labelled with ^{99m}Tc. We also estimated the plasma volume with ^{51}Cr red blood cells. The capillary permeability was determined by the difference in radioactivity of plasmatic ^{99m}Tc albumin between 10 and 60 min. Measurement at 75 and 100 min showed a considerable variability, since in the same subject radioalbumin is already recirculating from the lymphatic system. In each subject we injected ^{99m}Tc albumin (0.1 Ci/kg). This quantity enabled us also to exclude subjects with positive joint scintigram.

In rabbits the evaluation of capillary permeability was carried out by the same method. We employed human radioalbumin because species specificity is not necessary in permeability studies [1, 2]. Blood was withdrawn by a polythene catheter placed in an iliac artery. In rabbits under anaesthesia (Nembutal 25 mg/kg) the abdomen was opened and bleeding was stopped by thermocauterization of small cutaneous vessels and then an iliac artery was cannulated. The radioactivity of the plasma and the urines was determined by well type scintillation counter SELO model CS2.

Results

Human subjects. The capillary permeability varied greatly in different groups of subjects (fig. 1). In controls the decrease of ^{99m}Tc albumin at

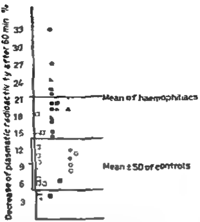

Fig 1 Capillary permeability in controls and in subjects with diseases of the haemostasis, □ = Controls ● = haemophiliacs A, ▶ = haemophiliacs B △ = afibrinogenaemics, * = congenital lack of factor V, ○ = ITP, ■ = von Willebrand s disease

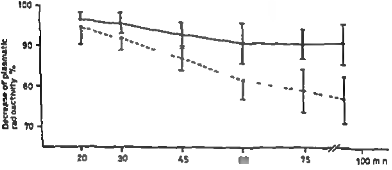

Fig 2 Plasmatic radioactivity at different times in controls (○) and in haemophiliacs (●)

60 min was 9.3 ± 4 6%, whereas haemophiliacs showed an average decrease of 21 1 ± 6 1%. The behaviour of plasmatic radioactivity at various times after radioalbumin administration is shown in figure 2 The two afibrinogenaemics showed a similar behaviour (20 7 and 19 8, respectively), on the contrary, subjects lacking of factor V were in the range of controls

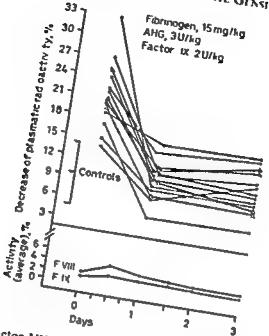

Fig 3 Effects of factor VIII and fibrinogen administration on capillary permeability ● = Haemophiliacs A, ▲ = haemophiliacs B, △ = afibrinogenaemics

Among the 3 patients with von Willebrand's disease, only the patient with a factor VIII level of about 15% showed a decrease of radioalbumin of 19%. The other 2 patients with a factor VIII level of about 60% showed values of capillary permeability.

The administration of 3 U/kg of factor VIII fully corrected the permeability in all haemophiliacs and in the patient with von Willebrand's disease. The correction persisted for 3 days after administration of factor VIII, at this time there was no remaining factor VIII in plasma (fig 3). The administration of 15 mg/kg fibrinogen to afibrinogenaemics corrected completely the permeability (fig 3). Factor VIII and fibrinogen administration did not modify the capillary permeability in controls.

The correction of the capillary permeability did not depend on the coagulation defect, which remained unaltered. We did not observe any change in thromboelastographic times after administration of 1 U/kg factor VIII or 2 U/kg factor IX in the factor VIII level, in activated partial thromboplastin time and in the prothrombin consumption index. The administration of 3 U/kg corrected the coagulation defect only for 1–3 h (normal values of TEG), whilst the correction of the permeability persisted for at least 3 days.

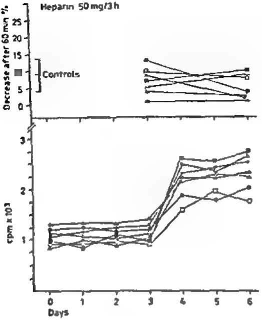

Fig 4 Effects of heparin administration on capillary permeability and microhaematuria The upper part of the figure represents capillary permeability the lower part represents microhaematuria

Heparin administration did not significantly change the capillary permeability to radioalbumin, whilst we observed in all subjects a significant increase in microhaematuria (fig 4)

Rabbits The administration of heparin and dicoumarol did not modify the capillary permeability to ^{99}Tc albumin Dicoumarol treated animals did not show spontaneous haemorrhages On the contrary, we observed severe mucous and internal haemorrhages in 3 rabbits treated with heparin 2 of which died

Discussion

Our results showed a more rapid decrease of plasma radioalbumin in haemophilia and in afibrinogenaemia than in controls We found no changes of plasmatic volume during the performance of the permeability test Therefore the rapid decrease of plasmatic radioactivity appears to be

related only to a real increase in capillary permeability. On the other hand the negative joint scintigrams ruled out any increased permeability localized in joints previously subjected to haemarthroses. Therefore the capillary permeability to proteins of low molecular weight, as albumin, is increased in haemophilia and in afibrinogenaemia. WITTE and BRESSEL [22] found an increased microvascular permeability when they observed that cantharidin blisters in haemophilia contained a greater activity of coagulation factors (factor V, VIII and II) than in controls. Considering our results and those of WITTE and BRESSEL we draw the conclusion that a modification exists in the 'barrier' function of the microvascular wall in haemophilia and in afibrinogenaemia.

The mechanism(s) causing the increased permeability is not clear. The common element of haemophilia (A and B) and afibrinogenaemia should be expected to be a reduced production or absence of the fibrin film, the existence or at least a function of which, is ascertained by many authors [5, 6, 8, 16]. However, the coagulation disorder *per se* seems not likely to be sufficient to cause an increase in capillary permeability. Neither heparin administration in man nor anticoagulant treatment in animals increased capillary permeability, although in man we observed a marked microhaematuria.

WITTE [21] observed an increased permeability to fluorescein in some rats (but not in all) after administration of very large doses of heparin, which contemporaneously caused also a severe bleeding. We think that these experiments are not comparable with ours, because of the difference in animals employed, the dosage and the method employed in evaluating capillary permeability.

We conclude that the increased permeability is not related to clotting defects and therefore to the absence of the eventual fibrin film, but to the specific lack of factors VIII, IX and I. We could hypothesize that antihaemophilic factors and fibrinogen could be involved in the composition of the microvascular wall and in its functions, namely normal permeability and normal containment of blood. In fact the administration of small doses of factor VIII and fibrinogen, insufficient to modify the coagulation defect, immediately corrects the increased permeability and this correction persists while there are no remaining quantities of factor VIII in plasma. This hypothesis is supported by the observation that factor VIII depletion in rabbits by goat anti-VIII immune serum increases the capillary permeability when factor VIII plasmatic activity has fallen below 5% from 48 to 60 h [17]. From these results we are unable to assert whether

the increased permeability facilitates the bleeding in haemophilia and in afibrinogenaemia Zweifac s [23] researches showed that hyperaemia is sufficient to determine ha— rhages in an experimental animal with increased capillary permeab or after heparin administration We observed [17] that in rabbits t with anti factor VIII goat serum the increased permeability precec orrhages by 12 h This delay could indicate that increased perm d bleeding are related not only to the clotting defect alone ranges of the vascular walls as a consequence of the deplc III but only after a long time

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Acta Haemat 52 345–348 (1974)

Antiglobulin Antibodies and Anticomplementary Factors in Hepatitis B Antigenaemia

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Abstract A study of an apparently healthy population of Southern African Negroes showed that the HB antigen carrier state may predispose these subjects to produce a higher incidence of antiglobulin antibodies and anticomplementary factors It is presumed that the anticomplementary factors represent excess HB antigen antibody complexes in a modified form while the antiglobulin antibodies are the host s humoral response to the modified complexes

Key Words
Anticomplementary factors
Antiglobulin antibodies
Hepatitis B antigenaemia

Of the various methods for determining hepatitis B (HB) antigen it is generally accepted that the complement fixation test [4] leads to the recognition of anticomplementary factors THIRY *et al* [7] found that such anticomplementary factors can be found in over 53% of patients with cirrhosis, 14% of patients with acute hepatitis and 7% of apparently healthy subjects known to be HB antigen carriers

The present study was designed to determine the incidence of anticomplementary factors in a population of Southern African Negroes who as a result of a combination of suboptimal socio-economic and tropical climate conditions, have been found to possess a high frequency of HB antigen [8] The incidence of antiglobulin antibodies was also investigated with a view to establish a possible relationship between such antibodies and the occurrence of anticomplementary factors

Materials and Methods

Sera were obtained from 360 HB antigen positive and 240 HB antigen negative Southern African Negroes The presence or absence of HB antigen virus was de

termined by the inhibition cross over electrophoresis method of MILNER et al [3] For the evaluation of anticomplementary activity we used the complement fixation method described by THIRY et al [6] in their study of patients with cirrhosis STEINBERG's [5] method was used to determine the presence of antiglobulin antibodies Sera which gave strong reactions when tested against anti Rh sensitized red cells were considered to possess antiglobulin antibody activity and were further examined by the haemagglutination test for their anti Gm or anti Inv specificities Antiglobulin antibodies that showed an inhibition pattern when tested against a panel of known Gm or Inv types were classified as 'specific' while those lacking identifiable specificities were classified as 'non specific' Dr ERNA VAN LOGHEM of the Netherlands Red Cross Blood Transfusion Service determined the Gm and Inv serum types of a panel of Southern African Negro bloods which we used for the haemagglutination inhibition test

Examples of incomplete anti Rh serum with unusual serological characteristics to bind complement C3 were also used in this study Red cells coated with such antibodies possess activities to agglutinate many known examples of anti Gm and anti Inv and almost always react against sera from rheumatoid arthritis patients

Results and Discussion

In the process of inactivating the labile serum complement factor it was found that undiluted serum can be heated for 1 h at 56 and 60 °C without causing significant changes to the electrophoretic pattern of the serum proteins This suggests that variations in the determination of anticomplementary activity, as a result of heat-induced changes, need not be different between samples inactivated at 56 and 60 °C However, serum heated at 60 °C for 1 h not only raised the incidence of anticomplementary activity among the non-carriers of HB antigen but more significantly increased its incidence among known carriers of the virus (table I) It appears that a substantial number of subjects with persistent HB antigenaemia possess a heat-induced variety of protein which readily consumes complement It is tempting to speculate that the heat-induced complement consuming factors represent a select variety of altered HB antigen-antibody configurations

Comparative antiglobulin antibody studies also showed that the incidence of so-called 'non-specific' varieties of antiglobulin factors was considerably greater among carriers of HB antigen than among the non-carriers (table II) No marked variations were observed with respect to the occurrence of 'specific' varieties of antiglobulin antibodies (anti-Gm or anti-Inv) A possible explanation for finding such a high incidence of non-specific antiglobulin antibodies could be that the majority of these

Table I The incidence of anticomplementary activity after heating undiluted sera of carriers and non-carriers of HB antigen for 1 h at 56 and 60°C

Subjects	Number examined	Anticomplementary positive sera observed at			
		56 C		60 C	
		n	%	n	%
HB antigen positive donors	360	16	4.4	115	31.9
HB antigen negative donors	240	7	2.9	26	10.8
χ^2 value	NS	NS		35.6 $p<0.001$	

Table II The incidence of antiglobulin antibodies among carriers and non-carriers of HB antigen

Subjects	Number examined	Allospecific antiglobulins (Gm or Inv)		Unidentified antiglobulins	
		n	%	n	%
HB antigen positive donors	360	6	1.7	[illegible]	22.5
HB antigen negative donors	240	4	1.6	31	12.9
χ^2 value		NS		9.1 $p<0.005$	

antibodies showed a marked preference to react against cells coated with a complex form of Rh antibody. Previous studies by us [9] established that cells coated with this type of Rh antibody possessed activities to bind complement C3, react with most rheumatoid factors and a variety of known reference anti Gm and anti Inv serum. In the absence of detailed information about the physico-chemical characteristics of the complement binding anti Rh, we postulate that cells coated with this type of immunoglobulin recognize antibodies to altered antigen antibody configurations.

In a further attempt to evaluate the nature of the so-called 'non specific' antiglobulin antibodies we found that in most cases they were not mercaptoethanol resistant, indicating that such antibodies are almost always of the IgM class. From extensive inhibition studies we also learned that most of the 'non-specific' antiglobulin antibodies found among carriers of HB antigen were of the 'non inhibitable' variety [2].

Although little is known about the biological significance of antiglobulin antibodies it has been suggested by WALLER *et al* [10] that their presence may be associated with the catabolism of antigen antibody complexes In other studies by ASHE *et al* [1] it was shown that antiglobulin antibodies might play a significant role in the host s response to herpes simplex virus infections by their ability to neutralize the virus in the presence of complement Whether the non specific' antiglobulin factors described by us play a similar role in the pathogenesis of HB viraemia has yet to be determined

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Acta Haemat 52 349-355 (1974)

Myeloma in a Case of Hodgkin's Disease

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Abstract This report describes the clinical and pathological features of a case of nodular sclerosing Hodgkin's disease treated with a variety of chemotherapeutic agents who developed fulminating IgG myeloma approximately 26 months after the initial diagnosis of Hodgkin's disease The possible significance of this association is discussed in relation to the previous literature concerning the development of a second malignancy in Hodgkin's disease

Key Words
Chemotherapy
Hodgkin's disease
Multiple malignancies
Myeloma
Paraproteins

It is now well recognised that a variety of haematological malignancies are associated with an increased chance of developing both a second haematological malignancy, and a range of non haematological tumours In this regard, Hodgkin's disease is probably no exception and the occurrence of second malignancies in this disease has now been the subject of a number of reports [1, 2, 4, 6, 9, 10, 12–14] For example, the occurrence of leukaemia in Hodgkin's disease is now well documented [15] However, myeloma in established Hodgkin's disease seems to be rare, and we have been able to find only one previous report of this association in which 2 patients with Hodgkin's disease treated with radiotherapy alone, developed typical myelomatosis [7]

The purpose of this paper is to report the occurrence and discuss the *features of a further case of undoubted Hodgkin's disease treated with al*kylating agents vinca alkaloids, and procarbazine, in whom fulminating IgG myelomatosis occurred 26 months after the initial diagnosis of Hodgkin's disease

Case Report

A previously fit Lincolnshire farmer presented in September 1971, at the age of 65 years with left supraclavicular fossa lymphadenopathy. A lymph node biopsy showed nodular sclerosing Hodgkin's disease, in which the plasma cell component was conspicuous and of normal morphology (fig 1). At this time, the peripheral blood count was entirely normal except for an ESR of 37 mm in 1 h. Bone marrow aspirate appeared normal, plasma cells made up less than 2% of all cells present and no abnormal forms were seen. Total protein level at this time was 7.5 g% (albumin 3.5 g%) and electrophoresis (fig 2) showed diffuse increases in the α_2- and β globulin regions together with a slight diffuse increase of γ-globulin; there was no evidence of monoclonal gammopathy. Abdominal lymphangiogram demonstrated probable pathological glands in the abdomen, but the liver and spleen scan was normal. A persistent fever was noted and he was, therefore, regarded as at least a stage III B disease. Further staging procedures were not performed because at that time multiple chemotherapy was regarded as the treatment of choice for both stage III B and stage IV disease.

Treatment with 6 one monthly courses of MVPP (mustine, vinblastine, procarbazine and prednisone) finished in May 1972, by which time all clinical evidence of

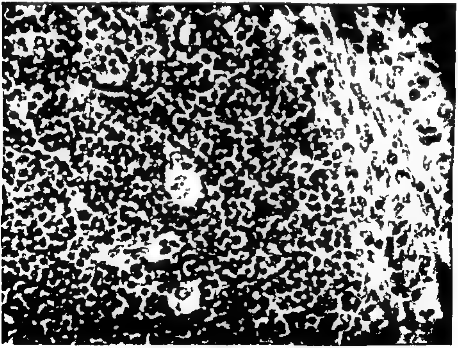

Fig. 1. Cervical node biopsy of September 1971. Part of one of the nodular areas is shown with fibrosis at one edge together with easily recognisable lacunar cells.

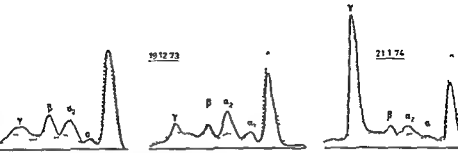

Fig 2 Changes in the electrophoretic strip Note the appearance of a tall monoclonal peak in the strip of 21 1 74 This paraprotein was shown to be IgG by immunoelectrophoresis – – – = Pooled normal serum

disease had d sappeared No further therapy was given until late July 1972, when the patient was started on continuous chlorambucil (5 mg day) This decision to give maintenance chemotherapy was based solely on the previous widespread disease. There was no cl nical evidence of recurrence and he remained well until mid October 1973 when he developed a chronic ulcer on the right tonsil This was biopsied and proved to be Hodgkin s disease The chlorambucil was increased to 8 mg day but the ulcer failed to improve he progressively lost weight, and suffered repeated episodes of severe pyrexia The patient s general condition gradually deteriorated and he was finally admitted to hospital for assessment in late December 1973

On admission the ulcer of the right tonsillar bed had now extended into both the pharyngeal wall and the palate Rubbery mobile nodes were palpable throughout both sides of the neck and in both supraclavicular fossae There were no other abnormal physical signs in part cular there was no other lymphadenopathy and no hepatosplenomegaly Haemoglobin 9 6 g% (having been 13 1 g% 2 months before) MCV [illegible] μm^3 MCH 37pg MCHC 33% WBC 3 100 mm^3 with a normal d fferential platelet count [illegible] 000 mm^3 ESR 140 mm in the first hour Sternal aspiration revealed a marrow in which cellularity M F ratio and granulopoiesis were all normal although erythropoiesis appeared dysplastic in some areas. There was a slight increase in reticulum cells Occasional abnormal looking bi and tri nucleated plasma cells were present but plasma cells made up only 3.5% of the entire population Serum electrophoresis (21 12.73) showed an increase in α_2 globul ns together with a small increase in the γ-region (fig 2) total proteins 6 3 g%, with an albumin level of 2.8 g% Immunoglobulin levels IgG 1 450 mg% (NR 800–1,800) IgA 220 mg% (NR 50–400) IgM 85 mg% (NR 50-200) Urea electrolytes, and liver function tests were normal Chest X ray showed normal lung fields, and no evidence of mediastinal or hilar gland enlargement Culture of the tons llar ulcer was sterile

In view of the clearcut recurrence of Hodgkin s d sease which had failed to respond to an increased dose of chlorambucil this drug was stopped and a further course of MVPP (the seventh *in toto*) started (19 12.73) However after the second injection of mustine and vinblastine (26.12.73) peripheral leucopenia (1 000 mm^3)

Fig 3 Marrow aspirate of 24 1 74 extensive replacement of the marrow with plasma cells of varying maturity

and thrombocytopenia (28,000 mm³) were noted and cytotoxic therapy was withdrawn Until his death approximately 1 month later (28 1 74) he remained pancytopenic the WBC count being between 1 000 and 1,500 mm³ with 80 90% neutrophils the platelets ranging from 40 000 to <10 000 and the haemoglobin falling progressively Despite vigorous supportive measures with broad spectrum antibiotics blood and platelet transfusions he deteriorated and remained pancytopenic despite both prednisone and oxymetholone therapy

During this period of pancytopenia routine liver function tests showed a progressive rise in total serum protein levels and this prompted serum electrophoresis (21 1 74) which demonstrated a very tall monoclonal peak in the globulin region (fig 2) Immunoelectrophoresis revealed the paraprotein to be IgGK immunoglobulin Immunoglobulin levels IgG 5 5 g% IgA 95 mg% IgM 40 mg% No Bence Jones protein was demonstrated Bone marrow aspiration (24 1 74) showed a cellular marrow extensively replaced with 60 70% of plasma cells including significant numbers of abnormal vacuolated and multinucleated forms (fig 3)

On the day following the marrow aspiration (25 1 74) the patient collapsed suddenly and was hypotensive a clinical diagnosis of gram negative septicaemia was made although blood culture was subsequently sterile Treatment with massive doses of parenteral broad-spectrum antibiotics and corticosteroids (solumedrone) was

instituted After initial improvement, a sudden massive haemoptysis occurred on 28 1 74, after which he became more hypotensive and died a few hours later

At *post mortem* there was moderate lymph node enlargement The largest nodes (up to 2.5 cm long) were in the neck and alongside the abdominal aorta, they were fibrous, yellow grey, and tended to form matted plaques Histologically, these were mainly collagenous, or showed areas of recent necrosis At their periphery was a zone of plasma cells Smaller, softer, nodes in axillae, inguinal regions and the porta hepatis showed total replacement by plasma cells, which were numerous in the moderately enlarged spleen (370 g) where normal lymphoid tissue was effaced

The bone marrow was pale throughout and in places rather soft, with at least two slightly gelatinous areas (less than 0.5 cm in diameter) in the strip of vertebral bodies examined Microscopically, the marrow was populated almost exclusively by plasma cells Most of the other macroscopically normal organs showed plasma cell infiltration The plasma cell morphology throughout was slightly abnormal, and larger primitive cells were noted

The lungs were bulky and abnormally firm, they contained extensive intra pulmonary haemorrhage and several yellow grey nodules (up to 1 cm in diameter) Histologically these proved to be aspergillomas, the fungus having eroded the walls of blood vessels in the area and also being found in the bone marrow Bacteriological cultures of the lungs and spleen were sterile

Death was attributed to terminal intra pulmonary haemorrhage and blood borne dissemination of pulmonary Aspergillus with myeloma as the underlying disease A detailed search of numerous sections failed to reveal evidence of residual Hodgkin's disease

Discussion

Although paraproteinaemia alone has been reported in Hodgkin's disease [5], this patient developed true myeloma as indicated by the presence of a massive serum paraprotein level, partial immune paresis, and heavy marrow infiltration by abnormal plasma cells To the best of our knowledge, the development of myeloma in Hodgkin's disease has previously been described in only two other patients [7]

In view of the apparent rarity of the association reported here, its significance must remain in doubt The risk of developing myeloma may be a hazard of the Hodgkin's disease itself, it may be a hazard of the immunosuppressive and carcinogenic regimes used to treat the disease, alternatively the case may represent the chance association of two uncommon malignancies Of these three possibilities, the data relating to our case only allow comment about the possible role of chemotherapy in the genesis of the myeloma Our patient was given considerable amounts of chlorambucil, mustine, procarbazine and vinblastine, but no radiotherapy These drugs are known to be both immunosuppressive and carcinogenic,

and it is tempting to implicate them in both the causation and the fulminating course of the myeloma in our patient. However, in a recent large series of Hodgkin's disease, DE VITA *et al.* [6] found no increased incidence of second malignancies in the group of patients treated with MOPP (mustine, vincristine, procarbazine and prednisone) alone, and these workers showed that the increased risk occurred mainly in the patients treated with both radiotherapy and MOPP chemotherapy. Moreover, both the patients described by GREENBERG *et al.* [7] received only radiotherapy before the development of myeloma, chemotherapy being given only after the onset of the myeloma. Thus, in our patient, no final conclusion is possible about the role of the chemotherapy in the genesis of the myeloma.

There is now some evidence that the malignant clone of plasma cells in myelomatosis initially emerges anything up to 20–30 years before clinical presentation [8]. If this is true then our patient may have been living 'symbiotically' with his myelomatosis for many years without any clinical or laboratory evidence of it. Something happened in the last 6–8 weeks of his life to accelerate the progress of his myelomatosis. This might possibly have been the relapse of his Hodgkin's disease or the therapy for that relapse.

We must be cautious, however, in our speculation and the fact remains that this association between Hodgkin's disease and myeloma may have been due to chance alone.

Acknowledgements. We should like to thank Prof. F. G. J. HAYHOE for advice and encouragement and for his permission to publish this case. We are also indebted to Dr. G. FARRER BROWN of the Bland Sutton Institute of Pathology and Dr. M. H. Bennett of the Pathology Department, Mount Vernon Hospital, for confirming the diagnosis of Hodgkin's disease. Thanks are due to Mrs. V. WASHBOURN for typing the manuscript. J. C. C. is in receipt of a Leukaemia Research Fund Research Fellowship.

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Acta Haemat 52 356–361 (1974)

Thrombotic Thrombocytopenic Purpura with Autoimmune Pancytopenia

A Case Report

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Abstract A typical case of thrombotic thrombocytopenic purpura (TTP) with fatal course characterized by hemolytic anemia thrombocytopenia and leukopenia with neutropenia is described An autoimmune process against blood cells is proved by the presence of autoantibodies of the immunochemical type IgA+C fixed to erythrocytes complete and incomplete platelet antibodies and cytotoxic leukocyte antibodies On the basis of autoimmune disorders associated with TTP and the presence of the vascular thrombosis in some immunological reactions this case of TTP could be ascribed to the presence of blood cell autoantibodies

Key Words
Autoimmune diseases
Blood cell autoantibodies
Hemolytic anemia
Pancytopenia
Purpura
Thrombotic thrombocytopenic purpura

Thrombotic thrombocytopenic purpura (Moschowitz's syndrome) is a complex disorder whose etiology is unknown and of uncertain nosologic classification It is characterized by hemolytic anemia, hemorrhagic thrombocytopenia neurological symptoms, renal insufficiency and diffuse fibrin thrombi at the arteriolar and capillary level

Hematologic features have been ascribed to different causes The hemolytic syndrome, which is clearly extracorpuscular [19] seems to be due to erythrocyte damage produced by the contact with filaments of fibrin thrombi which partially occlude the arteriolae This hypothesis is supported by the data of BRAIN *et al* [7] Autoimmune pathogenesis of anemia is excluded on the basis of uncommon findings of erythrocyte autoantibodies [4 15 23] According to AMOROSI and ULTMANN's [3] report on 126 cases from the literature and from their own casuistic only 6% had a positive direct Coombs test Thrombocytopenia is ascribed to

coagulation defects, leading to an increased platelet consumption, as evident in the cases with massive thrombosis, or to damage suffered by the platelets at the level of the thrombi and of the endothelial lesions Tests for platelet antibodies have been attempted in a few cases and thromboagglutinins have rarely been found [1, 22] Leukocyte count is normal or increased with neutrophilia, leukopenia is a rare finding [5, 8] The present paper reports on a case of thrombotic thrombocytopenic purpura (TTP) with hemolytic anemia, thrombocytopenia and leukopenia showing autoimmune features

Case Report

A 35 year old Sard man man was admitted to the Policlinico Gemelli on May 8 1973 with a negative family and personal history Since December 1972, the patient presented recurrent neuropsychic symptoms (headache visual changes lower limb paresthesia muscular asthenia, mental confusion and depression) Sometimes he was affected by abdominal pains and jaundice Laboratory findings showed anemia and high indirect bilirubinemia, while neurological and electroencephalography tests gave negative results

On admission physical examination revealed pallor and jaundice The patient
pres n ed alternatively drowsiness and agitation with no signs of neurological focal
les [illegible] His temperature was 38 °C. The respiratory apparatus was normal.
Rhy [illegible] tachycardia was present blood pressure was 150/90 The liver was palp-
able [illegible] d the spleen 5 cm below costal margin

O [illegible] on the Hb was 5.4 g%, erythrocyte count 2 200 000/μl reticulocyte
count [illegible] cleated cells 12,300 μl with 8 400 acidophilic and polychromato-
philic [illegible] sts leukocytes 3 900/μl with neutrophils 65%, lymphocytes 29%
mono [illegible]
ous ery [illegible]
gated [illegible]
190 000 [illegible] Bone marrow aspiration revealed erythroid hyperplasia and a
normal [illegible] gakaryocytes Total serum proteins 6.8 g% Serum electro-
phoresis [illegible] α_1 globulin 6.5% α_2 globulin 10.3% β globulin 15.8%
γ-globulin [illegible] mmunoelectrophoresis was normal SGOT 51 U/l SGPT
11 U/l LE [illegible] s were negative Serological test for syphilis was nega
tive Bloo [illegible] se [illegible] blood urea nitrogen 37 mg% EEG showed nonspe
cific diffus [illegible] bral da [illegible]

Immuno [illegible] ol g [illegible] were performed as follows antierythrocyte anti
bod es with [illegible] at nat [illegible] line direct Coombs test with antigammaglobulin
anti IgG ant [illegible] anti Ig [illegible] anticomplement sera [10] ant leukocyte antibod
ies with cyto [illegible] test [19 [illegible] atelet antibodies with thromboagglutination test
[17] and antigl [illegible] n consump [illegible] est [24]

D rect Coo [illegible] est was per [illegible] IgA+C') ind rect Coombs test and aggluttna
tion with enzy [illegible] eated cells [illegible] negative Cold agglutinins were absent. The

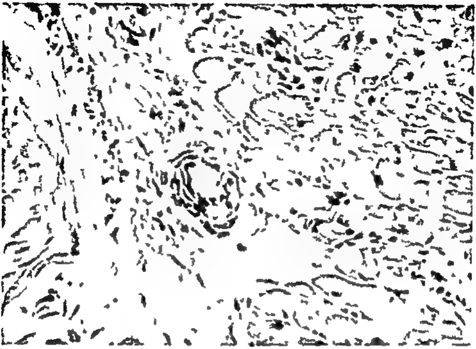

Fig 1 Histological aspect of cardiac arteriolar thrombosis

blood showed spontaneous hemolysis when incubated at 4 or 37 °C. Antileukocyte antibodies cytotoxic test was strongly positive for neutrophils and lymphocytes of 4 out of 6 samples tested Thromboagglutination test was strongly positive for 5 out of 6 samples tested Antiglobulin consumption test was positive

The patient was treated with steroid therapy and blood transfusions which produced a momentary recovery from neuropsychic symptoms But the general conditions were deteriorating and after some melena discharges he entered coma and died on May 16 1973

Necropsy showed high degree jaundice hepatosplenomegaly pulmonary edema hemorrhagic gastritis and cystitis meningeal sinus thrombosis with venous stasis cerebellar and cortical hemorrhages general lesions of the nervous tissue and nephrotic aspect of the kidney Histological examinations showed diffuse fibrin thrombi in arteriolae and capillaries of several organs especially in the kidneys heart and spleen (fig 1)

Discussion

The case described is a typical example of TTP with neuropsychic symptoms hemolytic jaundice hemorrhagic thrombocytopenic syndrome renal insufficiency diffuse fibrin thrombi at the arteriolar and

capillary level The patient, moreover, was affected by leukopenia with neutropenia together with anemia and thrombocytopenia showing a typical picture of pancytopenia, rarely described in the course of TTP The occurrence of erythrocyte-fixed antibodies (type IgA+C') complete and incomplete antiplatelet and cytotoxic antileukocyte antibodies reveal a possible autoimmune mechanism in determining pancytopenia. The probable pathogenetic correlation between blood cell autoantibodies and disseminated thrombosis is not easy to explain The TTP cases with positive Coombs test are relatively rare and, on the other hand, disseminated intravascular thrombosis is not a feature of autoimmune hemolytic anemia (AHA) or of thrombocytopenia and leukopenia due to autoantibodies

From the literature one could deduce that TTP is a syndrome rather than a disease [21] TTP seems to represent a complication of various diseases like malignant hypertension, sepsis, carcinomatosis and SLE [16, 11, 2], Sjogren syndrome [18], rheumatoid arthritis [6, 21] which is of particular interest as far as a possible relationship between disseminated thrombosis and immunological disorders is concerned

The concurrence of thrombotic phenomena and immunological reactions has been found in kidney transplant rejection in which a hemolytic anemia similar to that of TTP was also reported [12] The analogy between TTP and fibrin precipitation in Sanarelli-Shwartzman reaction has been also emphasized by TAUB *et al* [20] The deposition of immunocomplexes at the level of thrombotic formations in a TTP case [13] seems to be indicated by the fixation of IgM and complement shown by immunofluorescence

All these considerations support the hypothesis of a pathogenic correlation between disseminated intravascular thrombosis and immunological disturbances In our case, it could be a secondary fact due to the presence of blood cell autoantibodies and the TTP case with positive direct Coombs test could be considered AHA complicated by disseminated thrombosis, which in turn increases the pancytopenia by means of the above mentioned mechanisms

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Deutsche Gesellschaft für Bluttransfusion (Hrsg.) **Forschungsergebnisse der Transfusionsmedizin und Immunhämatologie.** Bericht des 15. Kongresses der Deutschen Gesellschaft für Bluttransfusion, Giessen 1972. Vol. 1. Medicus Verlag, Berlin. 429 pp., 105 fig., 86 tab.

Nach der einleitenden Karl Landsteiner Gedächtnis-Vorlesung, gehalten von J. J. VAN LOGHEM über das Thema «Chronische Virusinfektionen und Autoimmunkrankheiten», befasst sich der grösste Teil des Bandes mit Themen aus der Immunhämatologie.

Ein Abschnitt ist Arbeiten über Blutgruppenantigene und Antikörper gewidmet, darunter eine besonders interessante Veröffentlichung, die auf die Komplexität des Problems Erythrozytenladung/Suspensionsstabilität/Agglutinabilität hinweist. Ein weiterer Abschnitt befasst sich mit dem Au/SH-Antigen (heute Hepatitis-B-Antigen genannt), insbesondere mit dessen Bedeutung im Transfusionswesen. Im Abschnitt über das HL-A-System finden sich Übersichtsarbeiten sowie Arbeiten, die sich mit der Problematik der Bestimmungsmethoden und der Antiserenspezifität befassen.

Ein grösserer Teil ist dem Morbus haemolyticus neonatorum gewidmet, wobei als wichtigste Themen die pränatale Behandlung in Form von intrauterinen Transfusionen, die postnatale Therapie durch Blaulicht oder Phenobarbitalmedikation erwähnt werden sollen. Im weiteren finden sich Arbeiten über Laboratoriumsanalytik sowie Anti-D-Prophylaxe (u. a. Anti-D während der Schwangerschaft, nach Aborten, nach Geburt von Kindern mit D^u usw.).

In mehreren Artikeln werden die Methoden der Langzeitkonservierung durch Tieftemperaturen erläutert und miteinander verglichen. Die Blutspenderwerbung durch Motivierung potentieller Spender findet in einigen lesenswerten Aufsätzen Platz. Bei der Anwerbung von männlichen Spendern soll das «Heldenhafte» der Blutspende betont werden, während bei Frauen eher der Muttertrieb angesprochen werden soll, mit dem Hinweis, dass durch die Blutspende Leben erhalten werden kann.

Aus den verschiedenen Arbeiten über Screening-Untersuchungen bei Blutspendern kann entnommen werden, dass der Aufwand kaum die erhobenen Befunde rechtfertigt, besonders da bei verschiedenen Screening-Testen, die für grosse Untersuchungszahlen geeignet wären, noch zuwenig Kenntnisse des klinischen Wertes, insbesondere in bezug auf eine Prophylaxe, bekannt sind.

Der letzte Abschnitt des Bandes fasst Aufsätze aus verschiedensten Gebieten der Hämatologie zusammen. Der Band dürfte alle diejenigen interessieren, die auf dem Gebiet der Immunhämatologie oder des Transfusionswesens tätig sind.

E. PFLUGSHAUPT, *Bern*

RICHARD E. ROSENFIELD: **Immunohematology Syllabus.** Intercontinental Medical Book Corporation, New York 1974. 112 pp. US $ 7.75.

Das kleine, handliche Buch enthält eine überraschend grosse Fülle von interessanten und wichtigen Daten aus der Immunhämatologie. Diese sind zum Teil in tabellarischer Form zusammengestellt. Der Text ist im allgemeinen sehr kurz ge-

fasst und setzt Vorkenntnisse voraus. Auch sind für verschiedene Abschnitte, insbesondere das Kapitel über die Kinetik der Antigen-Antikörper-Reaktionen mathematische Kenntnisse erforderlich. Während sich die fünf ersten Kapitel mit den theoretischen Grundlagen befassen (Blutgruppen und ihre Vererbung, Mechanismen des immunologischen Zellabbaues, physikalische Grundlagen der Hämagglutinationsteste, Kinetik der Antigen-Antikörper-Reaktion) sind die beiden letzten Kapitel klinischen Problemen gewidmet. Zunächst werden die verschiedenen Typen der erworbenen hämolytischen Anämien besprochen, wobei auch der paroxysmalen Kältehämoglobinurie ein längerer Abschnitt mit historischem Exkurs gewidmet ist. Das Schlusskapitel trägt den Titel Bluttransfusionsprobleme und gibt eine knappe Übersicht über die wichtigsten Risiken. Auch ist der neueste Stand der Kenntnisse berücksichtigt. Dass sich bei dieser knappen Darstellung oft etwas überspitzte Formulierungen nicht vermeiden lassen, ist selbstverständlich. So ist z. B. der Radioimmunoassay als einzige genügend empfindliche Methode zur Erfassung des Hepatitis-B-Antigens erklärt und die Heparinbehandlung des Defibrinisierungssyndroms erhält die Bezeichnung «spezifische Therapie für dieses Problem».

Zwischen die verschiedenen Kapitel sind Krankengeschichten eingestreut. Die sich daraus ergebenden Fragen sind, besonders im Zusammenhang mit dem unmittelbar vorausgehenden Kapitel, meist sehr leicht zu beantworten, geben aber dem Autor zum Teil die Möglichkeit einer weiteren Präzisierung.

Gesamthaft hat das Büchlein beim Referenten einen zwiespältigen Eindruck hinterlassen. Während gewisse Abschnitte sehr wichtige Informationen in einer knappen und klaren Form enthalten, sind andere entweder nur schwer verständlich, oder – besonders was die klinischen Teile anbelangt – lückenhaft. Von den vier vom Autor gesteckten Zielen (Integration des Themas Immunhämatologie, Anregung zur Lösung noch offener Probleme, Darstellung der verschiedenen Aspekte in einer auch für Wissenschafter anderer Disziplinen verständlichen Art, Präsentation wichtiger Daten in leicht zugänglichen Tabellen) dürfte das zweite und vierte am ehesten erreicht sein. Die Auswahl der Literaturangaben beschränkt sich grossenteils auf ohnehin bekannte Standardwerke wie RACE und SANGERS «Blood groups in man» oder DACIE's «Hemolytic Anemias» und hilft deshalb dem interessierten Leser kaum weiter.

Das Buch kann somit nicht vorbehaltlos empfohlen werden, mag aber manchem Leser, vor allem dem nicht ausschliesslich immunologisch tätigen Hämatologen, interessante Anregungen geben.

U. BUCHER, *Bern*

Libri

Deutsche Gesellschaft für Bluttransfusion (Hrsg.): Forschungsergebnisse der Transfusionsmedizin und Immunhämatologie. Bericht des 15. Kongresses der Deutschen Gesellschaft für Bluttransfusion, Giessen 1972. Vol. 1. Medicus Verlag Berlin. 429 pp., 105 fig., 86 tab.

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Bearbeitet von G. BOEHM, Basel
(B) = Book Reviews – Buchbesprechungen – Livres nouveaux

Association française des Hémophiles

L'Association française des Hémophiles a créé le «Prix international de l'Association française des Hémophiles» destiné à récompenser les travaux de chercheurs sur la physiopathologie ou la thérapeutique de l'hémophilie. Son montant est de 15 000 F. Il sera attribué pour la première fois à Paris en juillet 1975, à l'occasion du congrès d'Hématologie. Les travaux devront parvenir avant le 1er mars 1975.

Pour tous renseignements, s'adresser à l'Association française des Hémophiles, Secrétariat du Prix, 6, rue Alexandre Cabanel, *F-75015 Paris* (France).

Jean Julliard Prize

The 5th Jean Julliard Prize, which was established by the International Society of Blood Transfusion in memory of its first Secretary General, will be awarded during the 12th International Congress of Blood Transfusion to be held in Helsinki (Finland) from July 27 to August 1st 1975. The prize is reserved for investigators under 40 years of age in recognition of recently completed scientific work related to blood transfusion. The value of the prize is SFr. 3,000.

In order to qualify, candidates must forward 5 copies of an unpublished manuscript or a recently published paper to the Secretary General, Dr. F. Josso, 6, rue Alexandre Cabanel, *F-75739 Paris Cédex 15* (France) *before the 1st of April 1975*.

The Jury for selecting the recipient of the Julliard Prize was designated during the last meeting of the Executive Committee of the International Society of Blood Transfusion in Amsterdam on August 25, 1974, and consists of the following members: Dr. F. Kissmeyer-Nielsen (Denmark), Dr. B. P. L. Moore (Canada), Dr. H. R. Nevanlinna (Finland), and Dr. S. Seidl (West Germany).

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(B) = Book reviews – Buchbesprechung – Livres nouveaux